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# THE PRECIPITATION OF NITROGENOUS SUBSTANCES BY ALKALINE MERCURIC REAGENTS, WITH SPECIAL REFERENCE TO URINE

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Freund and Fellner's experiments with mercuric chloride and sodium carbonate (1) led to a general belief that alkaline mercuric reagents precipitate practically all nitrogen from biological fluids. Patein and Dufau (2) suggested mercuric nitrate and sodium hydroxide for this purpose, and later Schöndorff (3) and Benedict and Osterberg (4) found such filtrates of urine to be nitrogen-free. However, Bardach (5), von Spindler (6), and others had shown that nitrate solutions could not be properly analyzed by the Kjeldahl method, a limitation confirmed by Everett and Sheppard (7). Gilbert and Baudouin (8) demonstrated the presence of nitrogen in Patein-Dufau filtrates by heating the evaporated filtrates with soda-lime. Recently West and collaborators (9, 10) found appreciable amounts of nitrogen in their filtrates of urine, made by precipitating approximately neutral solutions with mercuric sulfate and barium carbonate.

We have isolated from the filtrates of Benedict and Osterberg, Freund and Fellner, and West *et al.* considerable quantities of a crystalline substance, identified by its composition, melting point, and lactimide as hippuric acid (7). This led us to a detailed study of the precipitation of nitrogen compounds.

## EXPERIMENTAL

We used 30 per cent mercuric sulfate in 1:10 sulfuric acid solution and 60 per cent potassium hydroxide solution as reagents. The precipitating mixtures were made definitely, though slightly, alkaline to litmus, as advised by Gilbert and Baudouin (8). The



procedure proved to be more convenient than West's and the filtrates were much less dilute. The potassium sulfate formed is advantageous in the Kjeldahl determinations and does not interfere with most colorimetric sugar determinations (see Everett, Shoemaker, and Sheppard (11)).

To solutions of the nitrogenous substances, or their salts, containing approximately 1.4 mg. of nitrogen per cc., equal volumes of the mercuric reagent were added, followed by sufficient potassium hydroxide solution to make the mixture slightly, but definitely, alkaline to litmus. During the precipitation the mixtures were kept near 0°. They were filtered or centrifuged and the filtrates made slightly acid to litmus with sulfuric acid. The remaining mercury was removed by hydrogen sulfide or powdered zinc. Nitrogen was determined in these filtrates, except where indicated in Table I, by the Koch-McMeekin modification of the Kjeldahl method, followed by Nesslerization in the presence of gum ghatti.

The results indicate that many amines and amino acids, together with certain acid amides and cyclic compounds, remain in the alkaline mercury filtrates. According to Whitmore (12) the mercuric compounds of acid amides are usually soluble in water, but relatively few appeared in our filtrates. We prepared mercuric compounds of adipamide, caproamide, diethylformamide, dimethylacetamide, heptamide, myristamide, phenylacetamide, stearamide, and valeramide, which have not been previously described. The first four homologues of the mercuric acid amides of the fatty acid series are soluble in neutral and alkaline solutions. The higher homologues and mercuric aspartamide, oxamide, succinimide, and tartramide are quite insoluble. Mercuric acid amides are not decomposed to mercuric hydroxide by alkali. The acetamide, formamide, succinimide, dibasic amide, and aromatic amide compounds form colorless basic precipitates in alkaline solutions.

We noticed a relation between the structure of these substances and their degree of precipitation by alkaline mercury. The precipitation of nitrogenous compounds containing a  $\text{—CO—NH—}$  or  $\text{—CO—CH}_2\text{—NH—}$  nucleus seems to be rendered difficult and incomplete by the presence of methyl or acetyl groups in the vicinity of the nitrogen atom. Thus formamide and the higher homologues are completely precipitated, while the intermediate ones are partially soluble. With the nitrogen completely alkylated

# Everett, Sheppard, and Johnson

TABLE I  
*Approximate Per Cent of Nitrogenous Substances in Alkaline Mercury Filtrates*

Acid amides		Alkylamines*	
N, N-Diethylformamide	100	Dipropylamine	Much
N, N-Dimethylacetamide	85	Tripropanolamine†	"
Succinimide	60	Triethanolamine	"
N, N-Acetylmethylurea	55	Tributylamine	"
Acetamide	35	N-Acetylglucosamine†	"
Butyramide	35	Isobutylamine	"
Salicylamide	15	Butylamine	Present
Phenylacetamide	T.	Diisoamylamine	"
$\beta$ -Phenylpropionamide	"	Dibutylamine	"
Propionamide	"	Betaine	"
o-Toluamide	"	Dimethylamine	"
Valeramide	"	Isopropylamine	"
Adipamide	0	Sec. butylamine	"
Asparagine	0	Diamylamine	"
Benzamide	0	Ephedrine	"
Caproamide	0	Propylamine	"
N, N-Dibenzoylethylene-diamine	0	$\beta$ -Dimethylaminoethyl alcohol	"
Ethyl carbamate	0	Triisoamylamine	"
Formamide	0	Isoamylamine	"
Furoamide	0	Methylamine	T.
Heptamide	0	Trimethylamine	"
Malonamide	0	Amylamine	"
Methyl urea	0	Ethylamine	"
Myristamide	0	Propanolamine	"
Oxamide	0	Triacetoneamine	"
Stearamide	0	Triamylamine	"
Thioacetamide	0	Tripropanolamine	"
Urea	0	Ammonia	0
Alkylamines*		Cadaverine	0
		Creatine	0
		Creatinine	0
		Dicaprylamine	0
		Epinephrine	0
		Ethanolamine	0
		Ethylenediamine	0
		Glucosamine	0
		Guanidine	0
		Histamine	0
		Methylguanidine	0
		Putrescine	0
		Tribenzylamine	0
Diethanolamine	100		
Diethylamine	100		
Tetramethylammonium hydroxide	100		
Triethylamine	100		
Acetylcholine	Much		
Choline	"		
Tetraethylammonium hydroxide	"		

TABLE I—*Continued*

Purines		Amino acids	
Caffeine	85	Leucine	0
Theophylline	45	Lysine	0
Theobromine	T.	Tryptophane	0
Adenine	0	Tyrosine	0
Guanine	0	Acylated amino acids	
Nucleic acid	0	Hippuric	95
Uric acid	0	N-Benzoylalanine	85
Xanthine	0	Acetyl- <i>m</i> -aminobenzoic	80
Amino acids		Glycocholic	70
Proline	90	N,N-Dibenzoylcystine	40
Aspartic	60	Taurocholic†	40
Oxyproline	60	Acetyl- <i>p</i> -aminobenzoic	25
Alanine	55	Arylamines	
Aminobutyric	55	Methylacetanilide	80
Sarcosine	55	Methylformanilide	70
Valine	55	Acetanilide	45
Aminomethylbutyric	50	Diacetyl- <i>p</i> -aminophenol	25
Glutamic	40	Acetyl- <i>p</i> -aminophenol	20
Isoleucine	40	<i>p</i> -Dimethylaminophenol*	15
Aminoisobutyric	30	<i>m</i> -Dimethylaminophenol*	15
<i>p</i> -Aminophenylacetic	25	$\beta$ -Hydroxyethylaniline	10
$\beta$ -Phenylalanine	25	Acetyl- <i>p</i> -methylamino-phenol	T.
N-Phenylglycine	25	<i>p</i> -Aminoacetophenone	"
Taurine	25	<i>p</i> -Aminophenol	"
Aminovaleric	20	Aniline*	"
Methionine	20	Cyclohexylamine	"
Phenylalanine	20	Diacetyl- <i>p</i> -phenylene-diamine	"
Cystine	15	Dimethylaniline*	"
$\alpha$ -Aminophenylacetic	15	Isovaleranilide	"
Glutathione	10	<i>o</i> -Methylaminophenol†	"
<i>m</i> -Aminobenzoic	T.	<i>p</i> -Phenylenediamine	"
Aminocaproic	"	Propionanilide	"
Aminocaprylic	"	Acetyl- <i>m</i> -aminophenol	0
5-Aminosalicylic	"	Acetyl- <i>o</i> -aminophenol	0
<i>o</i> -Aminobenzoic	0	<i>m</i> -Aminophenol	0
<i>p</i> -Aminobenzoic	0	<i>o</i> -Aminophenol	0
<i>p</i> -Aminophenylglycine	0	Tetramethyl- <i>p</i> -phenyl-enediamine*	0
Arginine	0		
Cysteine	0		
Glycine	0		
Histidine	0		

TABLE I—*Concluded*

Miscellaneous		Miscellaneous	
Nitric acid†	100	Allantoin	0
N-Acetylpyrrolidine†	55	Barbituric acid	0
Quinolinic acid	50	Bilirubin	0
Quinoline*	40	Biliverdin	0
Thiocyanic acid†	40	Cyanic acid†	0
Antipyrine*	25	2,5-Diketopiperazine	0
2,4-Dimethylquinoline*	25	3,5-Dimethylpyrazole*	0
Hexamethylenetetramine	25	N-Ethylpyrrole*	0
Piperazine*	20	Hydantoin	0
Hemin	T.	Hydroxylamine	0
Indole-3-propionic acid	"	2-Hydroxyquinoline	0
Methylisothiocyanate*	"	Indigotin	0
Parabanic acid	"	Indole	0
$\alpha$ -Picoline†	"	3-Methyl-5-pyrazolone	0
Piperidine*	"	Skatole	0
Pyridine*	"	Uracil	0
Pyrrole*	"		

T., trace (less than 10 per cent).

\* Kjeldahl method unreliable.

† Impure.

‡ Determined by specific methods.

very little precipitation occurs; instead these mercury compounds form mercuric hydroxide in alkaline solution. When both nitrogen atoms of urea are methylated or acylated much remains in the filtrate, but the presence of unprotected nitrogen, as in methylurea and urea, allows complete precipitation.

A majority of the alkylamines appeared in the filtrates. The Kjeldahl method fails to convert some of these, especially the lower homologues, quantitatively to ammonia. Hence the results for alkylamines are not always quantitative. Nevertheless, it is evident that there is less precipitation of the tri- and tetraalkylamines, including the betaines and cholines. The di- and triolamines, tetraalkylammonium hydroxides, betaines, and cholines do not appear to combine with mercury in alkaline solutions. Their double salts, formed in acid solution, are decomposed by alkali. The simple alkylamines form rather soluble mercury compounds. Those of the monoalkylamines are partially precipitated by alkali as colorless basic compounds, with the liberation of some free amine.

Di- and trialkylamine compounds are easily decomposed by alkali to mercuric hydroxide. Note that N-acetylglucosamine appears in the filtrate. Riedel (13) has shown the mercuric salt of chondroitinsulfuric acid to be soluble in alkali. The effects of methylation are also apparent with the purines (see caffeine).

There are different opinions concerning the precipitation of amino acids by alkaline mercury. Malfatti (14) found them to be precipitated with difficulty by mercuric chloride and sodium carbonate. Neuberg and Kerb (15) claimed that mercuric acetate, sodium carbonate, and alcohol precipitated all amino acids, but they encountered difficulties with alanine, proline, and valine. Moreover, Lippich (16) found dicarboxylic amino acids, and Vickery (17) choline and betaine, in the filtrates.

Many amino acids appeared in our filtrates, and nowhere were the structural relations more apparent. Glycine and the higher homologues were quantitatively precipitated, while alanine and sarcosine formed more soluble compounds. Of the branched chain amino acids only leucine was entirely precipitated. The unfortunate selection of leucine and glycine for their experiments led Lustig and Speiser (18) to conclude that amino acids were quantitatively precipitated. The amino acids which Lippich and Neuberg and Kerb found difficult to precipitate appeared in largest amounts in our filtrates. We have prepared crystalline mercuric amino acid compounds, not described in the literature, from alanine, aminoisobutyric acid, aminomethylbutyric acid, aspartic acid, hippuric acid, isoleucine, methionine, oxyproline, proline, and sarcosine. They were all soluble in water, those of alanine, aminoisobutyric acid, oxyproline, proline, and sarcosine being very soluble. The only mercuric amino acid compounds that are easily decomposed by alkali to mercuric hydroxide are those of aminomethylbutyric acid, isoleucine, and taurine, and of the N-substituted amino acids, as the conjugated bile acids, hippuric acid, proline, and sarcosine.

In one respect the precipitation of the amino acids differs from that of other nitrogenous compounds. Here the alkaline mercury filtrates are often yellow, owing to colloidal mercury-amino acid complexes which partially precipitate upon standing. The state of these complexes evidently varies, since we have found only one-fifth as much alanine in the filtrate of urine as in aqueous filtrates.

The acylation of the amino acids greatly interferes with their precipitation, hippuric acid, bile acids, etc., remaining in both aqueous and urine filtrates. We have recovered from the alkaline mercury filtrates the following amounts of hippuric acid, added to urine as the sodium salt; 95 per cent by our method, 90 per cent by Freund and Fellner's method, and 85 per cent by the method of West *et al.* The greater precipitation of hippuric acid by the latter method is undoubtedly due to the low pH of the precipitating mixture, mercuric hippurate being less soluble in neutral or slightly acid solution.

The alkaline mercuric compounds of the arylamines are also more soluble when their nitrogen is alkylated or acylated. The presence of a phenol group or a second amino group leads to precipitation. These compounds are not decomposed by alkali. Among the heterocyclic compounds, pyrrole, quinoline, and piperazine derivatives are not entirely precipitated, while those of indole, pyrimidine, and purine are entirely precipitated unless their nitrogen is protected. The heme and bile pigments are found, together with proline, in the original mercury filtrates in large amounts, but are later precipitated by acid during the removal of the mercury.

We have determined the nitrogen in alkaline mercury filtrates of certain biological fluids. Approximately 20 per cent of muscle extract nitrogen, 30 per cent of liver extract nitrogen, and 70 per cent of ox bile nitrogen are present in the filtrates. To determine the smaller amounts of nitrogen in alkaline mercury filtrates of urine, we distilled our digests with Goebel's modification of Pregl's microdistillation apparatus (19), which was made entirely of Pyrex glass in order to diminish errors from dissolved alkali. Because of the large amounts of potassium sulfate in these filtrates we used not less than 0.5 volume of 50 per cent sulfuric acid and 1 cc. of 30 per cent hydrogen peroxide (superoxol) for the Koch-McMeekin digestion. The superoxol was added cautiously after a preliminary 5 minute digestion. Then the mixture was boiled another minute. The steam distillation of the digest was conducted for 5 minutes with a brisk current of steam at a rate sufficient to cause the separation of much potassium sulfate in the distilling flask. This was followed by 1 minute of washing with steam. The ammonia was received in N/70 hydrochloric acid solution, duplicates being titrated (with methyl red as indicator) and Nesslerized.

This method gave excellent checks and recoveries, together with very small blanks (approximately 0.05 cc. of N/70 alkali).

The difference between Nesslerization and titration was taken as a convenient estimate of the amount of alkylamine in the digest. This procedure is justified if the alkylamines do not themselves give colors with Nessler's reagent. Thompson (20) says that methylamine gives 11.8 per cent of the color of ammonia, but we found that very pure, freshly prepared solutions of methylamine gave less than 3 per cent. Di- and trimethylamines gave no appreciable color. The difference between titration and Nesslerization was approximately 5 per cent for the digests of our alkaline mercury filtrates of normal night urine.

In a long series of determinations we found the alkaline mercury filtrates of normal night urine to contain 0.025 to 0.090 mg. of nitrogen per cc., which represented 0.75 to 2.80 per cent, average 1.60 per cent, of the original urinary nitrogen. These filtrates were made from samples of urine diluted to an approximate nitrogen content of 8 mg. per cc. We found that the maximum concentration that can be safely handled by 1 volume of the mercuric reagent is 10 mg. of nitrogen per cc. of urine, but we employed a dilution to 8 mg. per cc. as a precautionary measure. West and Peterson (10) made no provision for this in their method, and their urine filtrates contained more nitrogen as a result. Even with dilute urine our filtrates contain less nitrogen. With very concentrated urines their filtrates contain 20 times as much nitrogen as ours. A second reason for incomplete precipitation in their method is the inadequate alkalinity. We have found that similar filtrates, made by the addition of barium hydroxide to definite alkalinity, contain no more nitrogen than ours.

We have fractionated the nitrogen of alkaline mercury urine filtrates as follows: Larger volumes of filtrate were prepared from mixed samples of normal night urine. Mercury was removed by hydrogen sulfide and much of the potassium sulfate by the addition of several volumes of alcohol. The filtrates were then concentrated under reduced pressure at temperatures below 50°, the pH being carefully maintained at 6.7 during this concentration. Aliquots of the concentrated filtrates were then analyzed for total nitrogen by the distillation method; for urea, uric acid, creatine, creatinine, ammonia, and amino acids by Folin's methods; and for

hippuric acid by continuous and exhaustive extraction with ether in an apparatus which allowed the cooling of the concentrate during the extraction.<sup>1</sup> The ether extract contained only traces of urea, for which corrections were made.

The largest fraction (approximately 50 per cent) of the filtrate nitrogen was found to be due to unprecipitated hippuric acid, which was not removable by reprecipitating. Approximately 20 per cent was urea nitrogen, 10 per cent amino nitrogen, and 2 per cent creatinine nitrogen, with only a trace of uric acid. Relatively more urea, uric acid, and creatinine were present in similar concentrates of West and Peterson's filtrates. No thiocyanate or nitrate could be found in the concentrates. The rest nitrogen (about 15 per cent of the total) was neither creatine nor acid amide.

#### SUMMARY

The behavior of mercuric nitrogenous compounds in alkaline solutions has been studied. The degree of precipitation seems to be related to the structure of the nitrogenous substances, less precipitation occurring with alkylated or acylated nitrogen. Many amino acids, alkylamines, acid amides, and certain heterocyclic compounds are found in the filtrates. Mercuric compounds of a number of acid amides and amino acids have been prepared.

A method of precipitation of nitrogenous substances from biological fluids is described, together with analytical procedures that allow the accurate determination of small amounts of nitrogen in the filtrates.

Approximately 1.6 per cent of urine nitrogen, 20 per cent of muscle extract nitrogen, 30 per cent of liver extract nitrogen, and 70 per cent of bile nitrogen remain in the filtrates. Hippuric acid is the chief nitrogenous constituent of the urine filtrate, but urea, amino nitrogen, and traces of creatinine and ammonia are also present.

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<sup>1</sup> We are indebted to Dr. H. A. Shoemaker for designing this apparatus.



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## THE NATURE OF THE SUGAR OF NORMAL URINE

### II. THE BEHAVIOR OF CARBOHYDRATES IN BROMINE WATER AND THE KETOSE OF NORMAL URINE

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Bromine oxidation has long been accepted as a means of distinguishing aldoses from ketoses but has not received the careful analytical investigation it deserves. The original analytical procedure of Votocek and Nemecek (1) was based on earlier discoveries by Hlasiwetz (2) and Kiliani (3) and upon determinations of the amounts of *l*-arabinose, *d*-galactose, *d*-glucose, *d*-levulose, *l*-rhamnose, and *d*-xylose remaining after 24 hours of bromine oxidation at room temperatures. Votocek and Nemecek concluded that the presence of 60 per cent, or more, of reducing sugar after 24 hours indicated a ketose. Subsequent studies of bromine oxidation (4-6) have added little analytical information.

Our interest was aroused by noting apparent difficulties in the oxidation of higher oligosaccharide impurities of dextrans (7), which we believed to be aldoses. Lenart (8) also noticed that dextrans were difficultly oxidized. We tested the validity of bromine oxidation as an analytical test by making a careful study of the rates of oxidation of as many pure carbohydrates as possible. We wish to acknowledge the kind cooperation of Dr. C. S. Hudson, of Washington, D. C., who provided several sugars for us.

#### *Methods*

1 per cent aqueous solutions of the carbohydrates were placed in glass-stoppered flasks and enough bromine added to insure the presence of a small excess of liquid bromine throughout the experiment. The mixtures were kept in the dark at a constant tempera-

ture of 25°. After definite intervals they were aerated with washed air to remove bromine. The acids were neutralized by adding measured amounts of silicate-free potassium hydroxide solution to pH 7, with a spot plate. Aliquots were then analyzed

TABLE I  
*Sumner/Folin-Wu Ratios of Glucose Equivalents*

Original sugar		After bromine oxidation (48 hrs.)		
Inulin oligosaccharide..	4.45-6.00	Glycogen.....	7.00	
Soluble starch ..	5.35	Dextrin.....	6.70	
Dextrin ..	4.30-5.00	Soluble starch.....	5.30	
Glycogen ..	3.85	Starch.....	4.80	
<i>l</i> -Rhamnose.....	2.65	Dulcitol.....	2.75	
<i>l</i> -Fucose.....	2.55	Glycerol.....	1.35	
Amylotriose.....	2.35	Gluconic acid.....	1.30	
Maltose.....	1.85	Nucleic acid.....	1.20	
<i>d</i> -Arabinose.....	1.75	Mannitol.....	1.05	
Lactose.....	1.70	Sorbitol.....	1.05	
<i>d</i> -Mannose.....	1.70	Inositol.....	0.85	
<i>d</i> -Ribose ..	1.65			
<i>d</i> -Mannoketoheptose...	1.50	At equilibrium		
Cellobiose ..	1.45			
<i>d</i> -Glucoheptose ..	1.40	<i>l</i> -Fucose.....	2.00	
<i>d</i> -Mannoheptose ..	1.40	<i>l</i> -Rhamnose.....	1.50	
<i>l</i> -Arabinose.....	1.35	<i>d</i> -Glucosamine ..	0.70	
<i>d</i> -Galactose.....	1.30			
Caramel.....	1.25	New glucose equivalents	Sum- ner	Folin- Wu
Gentiobiose.....	1.15	Amylotriose.....	0.84	0.36
<i>d</i> -Glucuronic acid....	1.15	<i>d</i> -Glucuronic acid...	0.92	0.80
<i>d</i> -Xylose ..	1.15	<i>d</i> -Mannoketoheptose .	1.00	0.67
$\beta$ , <i>d</i> -Glucose.....	1.00	<i>d</i> -Mannoheptose.....	0.83	0.59
<i>d</i> -Glucose.....	1.00			
<i>d</i> -Levulose.....	1.00			
<i>d</i> -Glucosamine.....	0.40			
Epinephrine.....	0.11			
Creatinine.....	0.07			
Uric acid.....	0.00			

in duplicate by both Sumner and Folin-Wu methods.<sup>1</sup> Each experiment was subsequently repeated by another analyst using a different sample of sugar and, whenever possible, from a second source.

<sup>1</sup> See Everett (31).

By using the two analytical methods, we secured more reliable checks and were able to take advantage of the Sumner/Folin-Wu ratio of glucose equivalents of sugars (designated S/FW). This

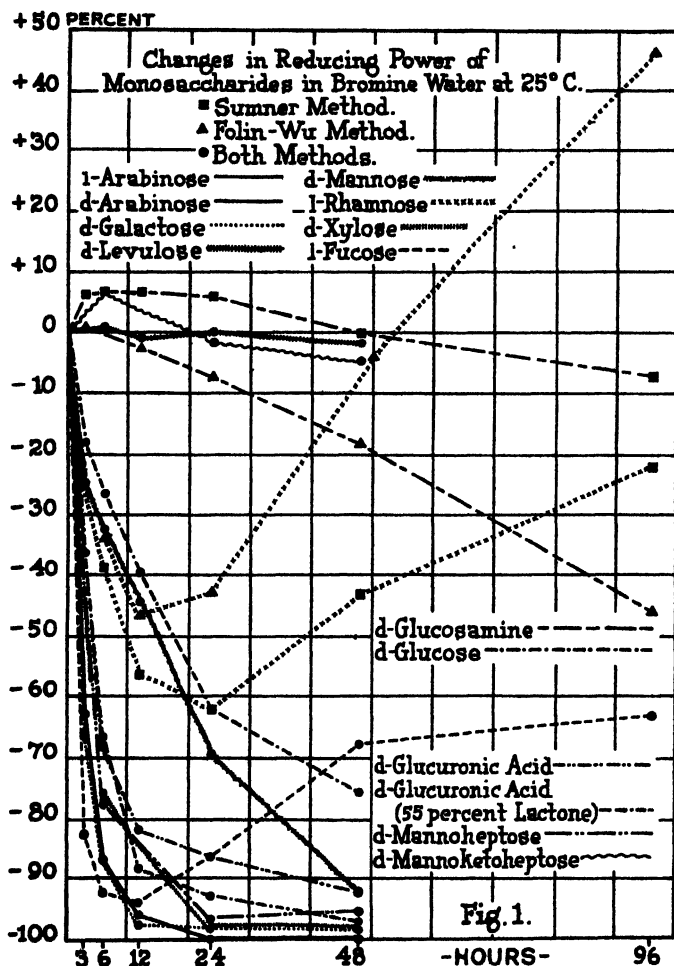


Fig. 1

ratio has been shown by Everett and Edwards (7) to be a helpful criterion for differentiating sugars. Known S/FW ratios are given in Table I, together with several newly determined glucose equiva-

lents. (Consult Poe and Klemme (9) for previous tabulations.) Our Folin-Wu equivalent for *d*-glucuronic acid is higher than that found by Quick (10). He used hydrochloric acid hydrolysates of

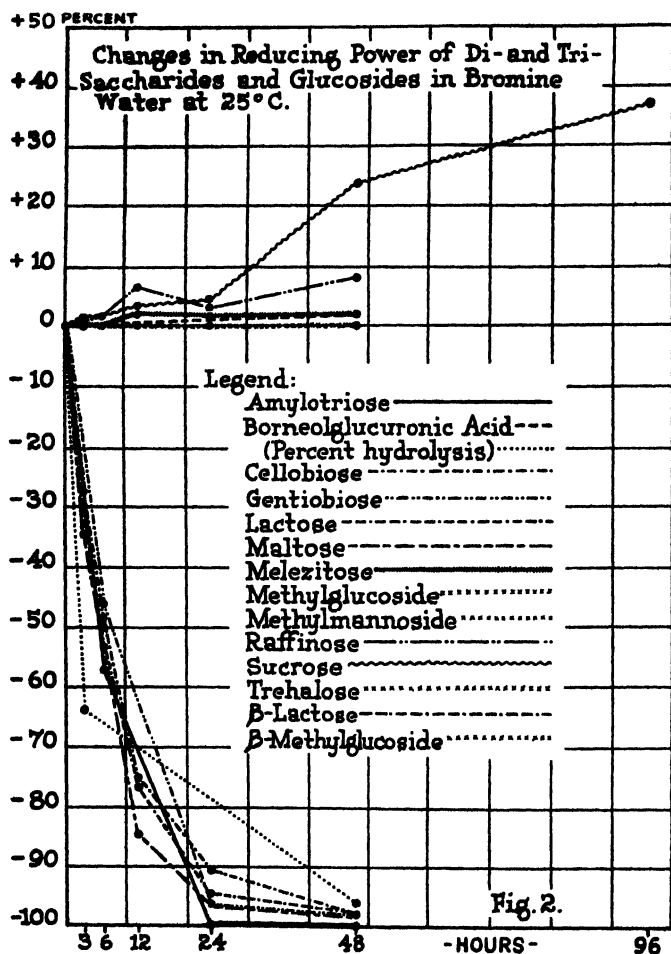


FIG. 2

mentholglucuronic acid, in which the sodium chloride, formed by neutralization, would cause low values (11).

Our results during the early hours of bromine oxidation are

TABLE II

*Effect of Bromine on Reducing and Non-Reducing Substances*

Reducing substances formed (as per cent glucose)		Reducing substances formed (as per cent glucose)	
Dulcitol (Sumner).....	24	Citric acid.....	0
Mannitol.....	24	Creatinine (Folin-Wu)....	0
Sorbitol.....	23	Gum arabic.....	0
Sucrose.....	23	Lactic acid.....	0
Inulin (Sumner).....	22	$\alpha$ -Methylglucoside.....	0
Starch.....	21	$\alpha$ -Methylmannoside.....	0
Glycerol (Sumner)*.....	19	Mucic acid.....	0
“ (Folin-Wu)†.....	14	Mucin.....	0
Soluble starch (Sumner).....	14	Urea.....	0
Nucleic acid (yeast)*.....	10	Uric acid.....	0
Dulcitol (Folin-Wu).....	9		
Dextrin (Sumner).....	8	Reducing substance remaining	
Inositol*.....	8		
Inulin (Folin-Wu).....	7		per cent
Glycogen (Sumner)†.....	6	d-Glucosamine (Sumner)...	100
Gluconic acid*.....	5	d-Levulose.....	98
Raffinose.....	5	l-Rhamnose (Folin-Wu)....	97
Starch (Folin-Wu)†.....	4	d-Mannoketoheptose.....	96
Soluble starch (Folin-Wu)....	3	d-Glucosamine (Folin-Wu) .	82
Galactonic lactone.....	2	l-Rhamnose (Sumner).....	57
Liebig's extract (Folin-Wu)...	2	N-Acetyl-d-glucosamine...	50
Melezitose.....	2	l-Fucose†.....	33
Dextrin (Folin-Wu)†.....	1.5	d-Glucuronic acid.....	7
Agar.....	1.5	d-Mannose.....	7
Saccharic acid.....	1.5	d-Mannoheptose.....	4
Borneolglucuronic acid.....	1	d-Ribose.....	3
Creatinine (Sumner).....	1	Cellobiose.....	2
Glycogen (Folin-Wu)†.....	1	d-Galactose.....	2
Ovalbumin.....	1	Gentiobiose.....	2
Pentanucleotide*.....	1	d-Glucose.....	2
Liebig's extract (Sumner)....	0.5	d-Xylose.....	2
$\beta$ -Methylglucoside.....	0.5	Lactose.....	1.5
Trehalose.....	0.5	$\beta$ -Lactose.....	1.5
Acetoacetic acid.....	0	Amylotriose.....	0.5
Artificial urine§.....	0	d-Arabinose.....	0.5
Artificial concentrate of alkaline mercury filtrate  .....	0	l-Arabinose.....	0.5

\* Sumner method; reduced at room temperature.

† Folin-Wu method; reduced at room temperature.

‡ Slight reduction at room temperature.

TABLE II—*Concluded*

§ Composition given by Everett and Sheppard (27), except that 3 cc. of 0.5 per cent lithium urate were taken in place of the sodium urate, and 1 cc. of a mixture of 0.1 per cent each of phenol, catechol, *p*-cresol, and hydroquinone in place of the *p*-cresol; also 1 cc. of 1 per cent sodium citrate added.

|| Contained 2.8 per cent sodium hippurate, 0.17 per cent urea, 0.03 per cent creatinine, and 0.04 per cent each of alanine, aspartic acid, cystine, glutamic acid, isoleucine, methionine, oxyproline, proline, phenylalanine, sarcosine, taurine, and valine (see Everett *et al.* (12)). The figures given in the table are for both methods unless stated. Acids were neutralized to slight acidity before addition of bromine, which was allowed to act for 48 hours at 25°.

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given in Figs. 1 to 5 as averages of the several determinations, wherever these agreed.<sup>2</sup> The abscissæ represent hours of bromine oxidation; the ordinates, per cent increase or decrease in the reducing power of the sugar. For the non-reducing sugars and polysaccharides, the ordinates represent per cent of conversion of non-reducing to reducing sugar (in terms of glucose).

#### DISCUSSION

In Figs. 1 and 2 it will be noticed that most aldoses are oxidized rapidly up to the 6th hour, the process slowing as equilibrium is approached. These are typical aldose oxidation curves. At 48 hours, less than 5 per cent of such aldoses remains unoxidized, the actual amounts being given in Table II.

*d*-Glucosamine is oxidized very slowly, so that it would be mistaken for a ketose by Votocek and Nemecek's method. Fischer and Tiemann (13) found that much *d*-glucosamine remained after weeks of oxidation, while only 20 to 40 per cent of the expected *d*-glucosaminic acid could be isolated. Glucosamine presents a second peculiarity in that the oxidation curves by the two analytical methods differ, whereas they are identical for the easily oxidizable aldoses. The increasing divergence indicates the formation of a new reducing substance whose S/FW ratio is much higher than that of glucosamine. We are attempting to isolate the substance

<sup>2</sup> While the present report is chiefly concerned with bromine oxidation of carbohydrates during the first hundred hours, we have also studied the phenomena over long periods of time. The interesting results obtained in this way will be given elsewhere because they are related to descriptive, rather than analytical, sugar chemistry.

and to determine whether other substitutions of the carbon atom (2), as by hydrogen in the 2-desoses, cause similar peculiarities of oxidation (see Bergmann *et al.* (14)).

We have always found *d*-mannose to be more slowly oxidized than other aldoses, although after 48 hours it cannot be mistaken for a ketose. The S/FW ratio remains unchanged throughout the experiment, indicating a slow rate rather than formation of new reducing substance. This peculiar behavior of *d*-mannose agrees well with the finding by Levene and coworkers (15) of similar difficulties with epichitosamine, which they have designated 2-amino-*d*-mannose. Alles and Winegarden (16) have shown that hypiodite also oxidizes *d*-mannose more slowly than *d*-glucose.

While *d*-glucuronic acid was oxidized rapidly, a mixture of 45 per cent free acid and 55 per cent lactone was oxidized more slowly. According to Kiliani (17) the lactone of mannohepturonic acid is also rather difficult to oxidize by bromine. The rates of oxidation of lactones and the related anhydro sugars need careful study. The published evidence for anhydro sugars is somewhat contradictory (15, 18). We were unable to secure *d*-galacturonic or *d*-mannuronic acid, but others report that these acids are easily oxidized (19, 20).

The aldomethylpentoses are rapidly oxidized during the early hours. Later there is a rapid and marked increase in the reducing power of the mixture, which reaches a maximum many days later. Remarkably enough, the reducing material formed from *l*-fucose has the same S/FW ratio as fucose. That from *l*-rhamnose has a much lower ratio, which is evidence of the formation of a different reducing substance. The fucose product is also a new substance, since it reduces the Folin-Wu reagent almost as much at room temperature as upon the water bath, a property which is common to dioses, trioses, certain tetroses, onoses, osones, and certain keto sugar acids. Kiliani (17) has prepared a similar substance, namely 5-keto-*l*-rhamnonic acid, or 1-methyl-*l*-arabinuronic acid, from *l*-rhamnose by nitric acid oxidation. Recently Votocek and Malachta (21) have prepared the same acid by bromine oxidation. We are now isolating the fucose derivative. Votocek and Nemecek's test will not distinguish between aldomethylpentoses and ketoses. To do this the entire course of the oxidation must be determined.



The two ketoses investigated were not appreciably oxidized by bromine in the early hours. A review of the literature indicates

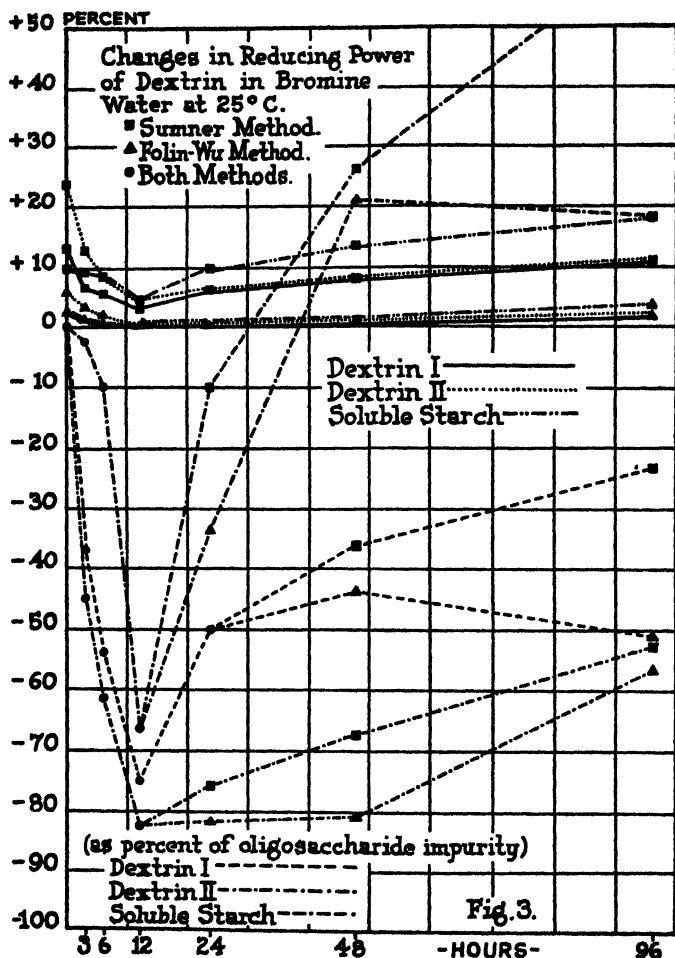


FIG. 3

that other ketoses and keturonic acids behave similarly. Only the aldehyde groups of osones are oxidized, 2-keto acids resulting (22).

The early effects of bromine upon dextrans and soluble starch are given in Fig. 3. The upper curves show the reducing materials in

terms of glucose. Note the high S/FW ratios of the original reducing oligosaccharide impurities. These substances are aldoses,

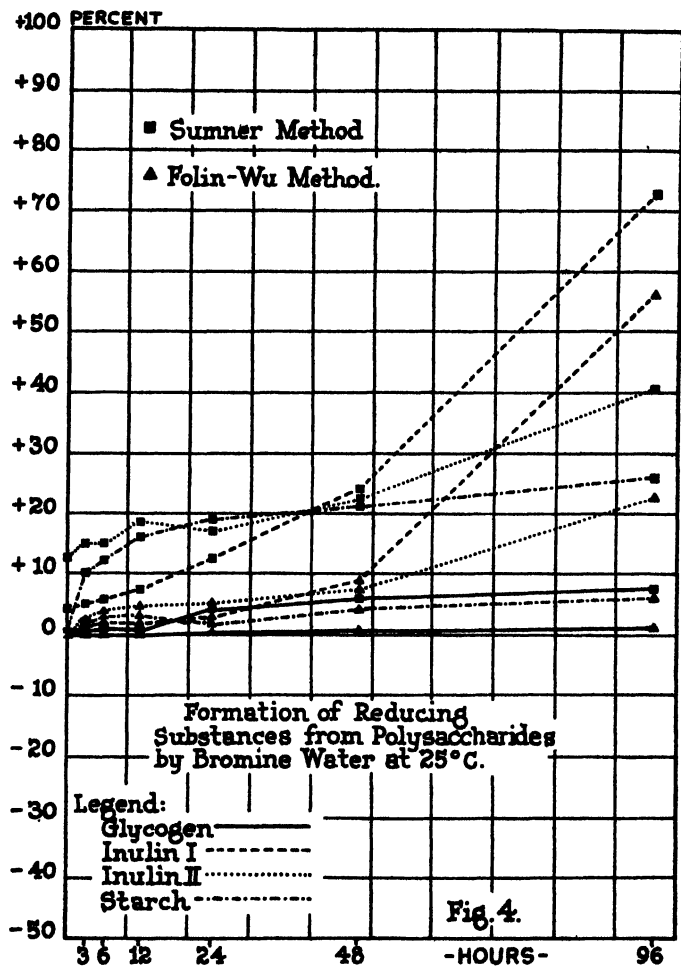


FIG. 4

because they are largely destroyed by the 12th hour. Then a new process sets in, as with the methylpentoses, resulting in a slow production of new reducing substances with high S/FW ratios. We are now studying the nature of these materials and comparing

them with the amyloextrinic acid of Syniewski (23). The higher reducing oligosaccharides will certainly be mistaken for ketoses by the customary bromine test, but may be detected by studying the entire oxidation curve.

Reducing substances with high S/FW ratios have also been produced from non-reducing polysaccharides by bromine oxidation (Fig. 4). Their formation from aldopolysaccharides was slow enough to prevent confusion with ketoses. Inulin, like sucrose (Fig. 2), was hydrolyzed quite rapidly but differed from the latter in one respect. Levulose was not the substance first formed from inulin because the two analytical methods gave diverging curves that became identical again after many days. From Fig. 2 it is apparent that borneolglucuronic acid is also easily hydrolyzed by the bromine treatment. Borneolglucuronic acid determinations were made by hydrolysis for 30 minutes on the boiling water bath with 1.2 N sulfuric acid. Only small amounts of reducing material were formed from this substance and from melezitose, the methylglucosides, raffinose, and trehalose.

#### *Effects of Bromine on Urine Sugars*

Bromine oxidation should aid in the characterization of the sugar of normal urine, which has remained unidentified since its discovery by Lespiau in 1848 (24). The presence of small fractions of glucose has been deduced by many from fermentation experiments, but has not been definitely proved. Recently West and Steiner (25) measured the rate of carbon dioxide production by yeast from concentrated alkaline mercury filtrates of urine. The small amounts of glucose found by them may have been produced during the concentration of the buffered acid filtrates. No evidence was presented to show that the hydrolyzable sugar of urine, also present in these filtrates, was not partially split to fermentable sugar. We have found the hydrolysis of urine sugars to occur with great ease, while Patterson (26) and others have shown that the hydrolytic products are fermentable. We have always found small amounts of fermentable sugar in urine but the major portion of the so called urine sugar is not fermentable. The failure of osazones to provide an answer to this problem has been considered by Everett and Sheppard (27). Benzoylation, which involves possibilities of extensive alteration and the inclusion of much nitrogenous impurity, has led to quite erroneous conclusions (28).

In preliminary reports (29, 30) Everett and Sheppard demonstrated that the reducing material of normal night urine is not appreciably destroyed by bromine in 48 hours and proposed the

TABLE III

*Effect of Bromine on Free and Total Sugar of Normal Urine*

The results are expressed as mg. per cent of sugar.

Sample No.	Method	Free			Total	
		Original	48 hrs. later*	After bromine	Original	After bromine
1	Folin-Wu	66	66	89		
	Sumner	64	64	55		
2	Folin-Wu	29	29	31		
	Sumner	32	32	32		
3	Folin-Wu	42	45	50		
	Sumner	54	58	48		
4	Folin-Wu	24	23	31		
	Sumner	24	25	32		
5	Folin-Wu	24	25	28		
	Sumner	26	26	29		
6	Folin-Wu	67	65	55		
	Sumner	72	74	57		
7	Folin-Wu	53		78	94	91
8	Folin-Wu	67		93	94	101
	Sumner	70		92	89	92
9	Sumner	20		23	27	28
10	Folin-Wu	51	51	71	87	86
	Sumner	50	52	66	65	71
11	Folin-Wu	24	22	26	42	29
	Sumner	24	25	29	32	30
12	Folin-Wu	37		42	54	42
	Sumner	42		42	55	46
13	Folin-Wu	21	22	27	33	
	Sumner	22	24	30	29	28
14	Folin-Wu	41	39	70	90	85
	Sumner	41	40	46	53	56
15	Folin-Wu	42	41	56	91	85
	Sumner	46	50	53	59	64

\* At room temperature, preserved with toluene.

name uroketose to designate the major, non-fermentable portion. Representative data supporting these conclusions are given in Table III. The methods of analysis have been previously de-

scribed (11, 31). To check the oxidation, pure *d*-glucose, *d*-glucuronic acid, *d*-levulose, and *d*-xylose were added to several of these urines. Over 90 per cent of the added aldoses was destroyed, but no levulose. The amounts of hydrobromic acid formed were evidently too small to cause serious interference with the Folin-Wu method because of the excellent agreement between the two methods, despite their differing sensitivity to bromides and chlorides (11).

The frequent increases of reducing power after bromine may be attributed to splitting of the hydrolyzable sugar by the acids generated during the oxidation, since the total sugar (free plus hydrolyzable) remained essentially unchanged. The easy hydrolysis of much of the hydrolyzable sugar establishes its similarity to glucuronates (Fig. 2). It can scarcely be the dextrin postulated by several investigators (28, 32, 33).

The bromine data are supported by experiments performed by Dr. Wayne M. Hull in this laboratory, and included by his permission in this manuscript. He studied the oxidation of sugars in urine by hypiodite, using an adaptation of Kolthoff's oxidation method, followed by the determination of unoxidized sugar by Sumner's method. He found that all added glucose, but only 30 per cent of the uroketose or of added levulose, was destroyed in urine. Incidentally, hypiodite also oxidizes many phenols and nitrogenous substances. Several years ago Fleury and Ambert (34) reported that hypiodite oxidized normal urine sugars completely, but they determined the amounts of iodine reduced by the urine, failing to consider that the Romijn-Willstätter method gives values for aldoses in urine that are much too high (35). Dr. Hull's results indicate that even ketoses reduce some hypiodite in urine. Hence Fleury and Ambert's conclusions are incorrect.

A much earlier confirmation of the ketose nature of normal urine sugar is found in the forgotten work of Friedländer (36). 70 years ago, with only qualitative tests at his disposal, he showed that this material was not destroyed by chlorine or iodine. At this early date glucose and lactose were the only reducing sugars available for experimentation, the isolation of levulose and the demonstration of its resistance to halogens being accomplished 15 years later. Hence, Friedländer concluded "das Zucker im normalen Harn nicht vorkommt."

We have also studied the bromine oxidation curves for urine, with the average results given in Fig. 5. The curve of the reducing material as it exists in normal night urines resembles that of a

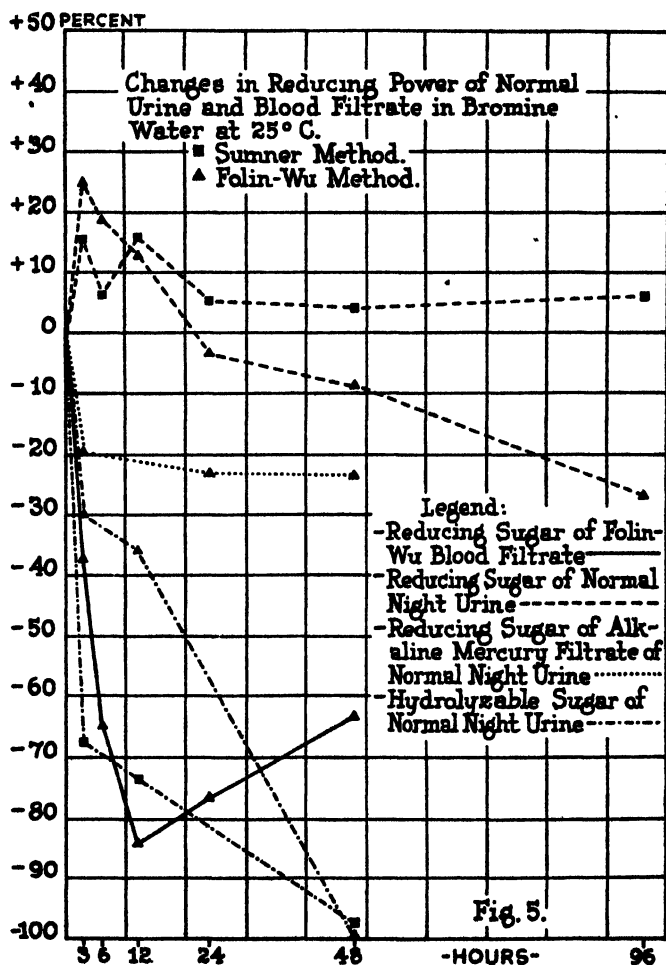


FIG. 5

ketose or aminoaldose. The presence of much easily oxidizable aldose is ruled out. In alkaline mercury filtrates (12) most of the material acted as a ketose, while approximately one-fifth resembled

an easily oxidizable aldose. A number of French investigators have continued to assume that the free sugar of normal urine is mostly glucuronic acid, a view which is definitely disproved by our results with bromine and the negative Bial's test of original urine.<sup>3</sup> While the naphthoresorcinol test is positive with practically all urines, it is also given by bromine-oxidized urines, although glucuronic acid added to urine is completely destroyed.

The curves also demonstrate the rapid splitting of hydrolyzable sugar, with the liberation of variable amounts of easily oxidizable sugar. The formation and subsequent oxidation of this material produced a peak in the curve during the early hours. It is peculiar that the easily split component cannot be detected in the dilute alkaline mercury filtrates. Alkaline mercury does not precipitate much of it because considerable amounts of hydrolyzable sugar have been found by us in concentrates of the alkaline mercury filtrates (12). It was easily split by bromine, resisted oxidation, and could not be removed by continuous extraction of the acid solution with ether. Benedict and Osterberg (38) have stated that glucuronates are precipitated by alkaline mercury, but we found one-third of pure borneolglucuronic acid and four-fifths of pure *d*-glucuronic acid in the filtrates. Neuberg and Mayer (39) have isolated very small amounts of the ether-soluble type of glucuronates from normal urine, but the identity of the major portion of the hydrolyzable sugar with glucuronates is very questionable. Neither can it be a dextrin because dextrans are precipitated by alkaline mercury and are difficultly hydrolyzable.<sup>4</sup>

When applying the bromine test to biological mixtures, it becomes necessary to consider possible errors from the production of new reducing substances by oxidation of non-sugar materials. We subjected many substances and mixtures, including artificial urine, to bromine oxidation, with the results noted in Table II. In addition, over 50 phenols were examined and found to contribute no additional reducing materials. Sugar alcohols and non-reducing ketose saccharides were the most likely sources of error. Cy-

<sup>3</sup> White and Green (37) speak of positive Bial's tests from normal urine, but they first partially hydrolyzed the urine with acids. Such tests have little analytical significance.

<sup>4</sup> Unpublished experiments of Edwards and Everett, in which dextrin was determined by hydrolysis and estimation of the reducing sugar formed.

closes and sugar acids caused smaller errors, while other substances were negligible. Relatively large amounts of sugar alcohols, sugar acids, or cycloses are necessary to falsify the bromine data. No substances of this nature, save minute traces of glycerol and cycloses, have been found in normal urine. Mixtures of known urinary constituents gave negative results. Normal urine contained no more fermentable reducing material after bromine oxidation than before; but the reducing substances formed by bromine, from sorbitol and mannitol added to urine, were entirely removed by yeast. Moreover, the urine curve does not show a rapid, early decline, followed by a slower incline, as would be expected if the reducing materials of Table II were replacing destroyed aldose. This destruction of free sugar and formation of new reducing material is, however, clearly demonstrated in tungstic acid filtrates of blood. The new substance formed here, after the 12th hour, may have its origin in the hydrolyzable sugar of these filtrates (11).

The bromine test indicates that the reducing material of normal urine is a ketose, keturonic acid, or aminoaldose. The latter would be precipitated by alkaline mercury unless its nitrogen were acylated or alkylated (12). We have been unable to detect such substituted amino sugars in the concentrated mercury filtrates by Ehrlich's test (see Mörner (40)). Whether the uroketose is a true sugar is uncertain, but it has been usually assumed to be so because of its analytical reactions. Its rate of destruction, when urine is evaporated to a small volume over an open flame, is intermediate between that of *D*-glucose and *D*-levulose (29, 41). A small portion of the urine sugar is precipitated by alkaline mercury and might therefore be nitrogenous, although this portion reduces both sugar reagents. Approximately a third is not destroyed by heating the urine with alkali at 100° (30, 42). Such alkaline digests no longer give the Tashiro-Tietz test (43) or colors with Bial's reagent on prolonged boiling, but still give naphthoresorcinol and Seliwanoff tests. The latter is very unreliable in testing urine for ketoses, since heating urine with hydrochloric acid alone produces a similar color. In our hands, Sampietro and Taufel's modification (44) gave no clearer information, since small amounts of added levulose could not be detected. The Tashiro-Tietz test was positive with normal urine, alkaline mercury concentrates, and



bromine-treated urines. While it is quite accurate and sensitive, we have found that not only furfural but also a number of aromatic aldehydes give this test. The generally unsatisfactory status of ketose color tests is further emphasized by our results with bromine-oxidized solutions of N-acetyl-*d*-glucosamine, dextrin, *l*-fucose, *d*-glucosamine, glycerol, *l*-rhamnose, soluble starch, and normal urine. After 96 hours of oxidation at room temperature, the urine mixture was the only one to give a Seliwanoff test or Tashiro-Tietz test. Oxidized urine and glucosamine both gave positive naphthoresorcinol tests. Very slight Bial's tests were given by oxidized dextrin and soluble starch. Colors in the Molisch test, in the order listed above, were brown, purple-red, brown-red, purple, green, brown-red, purple-red, and purple-red. Bergmann (45) has shown similar peculiarities for *dl*-lyxuronic acid. Tests for desoses were negative in alkaline mercury concentrates of urine.

We have long been impressed by certain similarities between phenols and reducing sugars, but the uroketose can be no ordinary phenol because of its great insolubility in ether at any pH. Moreover, the uroketose has a S/FW ratio near unity, while most phenols have very low ones and do not reduce powerfully enough at urinary concentrations. Millon and ferric chloride tests were uniformly negative in the alkaline mercury concentrates. We are now studying the separation of the reducing material from the nitrogenous substances of the alkaline mercury filtrates and the comparative chemistry of phenols and sugars. In this way we hope to characterize the uroketose more accurately.

#### SUMMARY

Quantitative studies of the action of bromine upon the reducing power of sugars and non-sugars have led to a new technique for the bromine test. The Sumner/Folin-Wu ratio of glucose equivalents assists in the identification of sugars. Lists of these are given, together with several new glucose equivalents.

Amino sugars, methylpentoses, and higher oligosaccharides have been shown to be easily confused with ketoses unless their oxidation curves are studied. The existence of new reducing derivatives of *l*-fucose, inulin, dextrin oligosaccharides, and *d*-glucosamine has been demonstrated. The oligosaccharide impurities of dextrans

and soluble starch proved to be aldoses. Certain structural relations of sugars have been considered.

The resistance of the major portion of the so called sugar of normal urine to bromine and hypiodite oxidation warrants the name uroketose. The general properties of this substance have been considered. On the other hand, a portion of the hydrolyzable sugar of urine is more easily oxidized. This material is easily hydrolyzed by acids and appears to differ from both dextrans and glucuronates, although small amounts of the latter are undoubtedly present. Ketose color tests have been discussed.

The major portion of the free sugar of tungstic acid blood filtrates is oxidized as aldoses are, but a second substance is present also.

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# AN IMPROVEMENT IN THE VAN SLYKE METHOD FOR BLOOD GAS ANALYSIS

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In his methods for blood gas analysis Van Slyke and coworkers (1-4) stress one difficulty: if  $\text{CO}_2$  and  $\text{O}_2$  are to be determined in the same sample, the blood proteins are precipitated by potassium ferricyanide, the lactic acid making the reaction of the solution acid. The clots which result do not interfere with reading when formed in the bulb, but when they occur in the calibrated portions, reading becomes impossible. By working quickly and by using the minimum amount of lactic acid necessary, one may succeed in partially overcoming these difficulties so that the calibrated parts are kept clean, but even then the clots will impair the exactness with which volumes can be measured in the apparatus.

By modifying the reagent we have found a way to avoid the clotting without making the analysis more difficult.

Our reagent consists of the following.

Potassium ferricyanide .....	3.3 gm.
Saponin.....	3.3 "
Urea.....	450.0 "
Octyl alcohol.....	5.0 cc.
Distilled water to.....	1000.0 "

The necessary quantity of lactic acid (0.66 cc. of N lactic acid to 6.0 cc. of reagent) is put into the cup of the apparatus immediately before the reagent is made air-free by evacuation.

Our reagent differs from that of Van Slyke only by the addition of urea. Since urea prevents protein precipitation even in acid solutions, by its use any interfering precipitation can be completely avoided. The high concentration of urea proved necessary

only for some kinds of blood, especially for sheep blood, while for others smaller quantities may be sufficient.

This reagent may be used for macro- and microanalyses of all blood gases in one sample and for determination of CO in the blood after absorption of the O<sub>2</sub> with pyrogallol or hydrosulfite. We also

TABLE I

*Comparison of Analyses Made with Modified Reagent and with Original Van Slyke Reagent*

*P* = volume of blood sample in cc.; *S* = cc. of total solution extracted in the gas chamber; *A* = volume of gas, in cc., at which the pressure was read.

Kind of blood	Reagent	Pressure read directly on manometer			
		CO <sub>2</sub>	O <sub>2</sub> + N <sub>2</sub>	O <sub>2</sub> *	CO + N <sub>2</sub>
		<i>P</i> = 1.0, <i>S</i> = 3.5, <i>A</i> = 2.0			
		mm.	mm.	mm.	mm.
Dog	New	141.5	101.5		
"	Original	140.0	103.0		
Human	New	190.0	68.0		
"	Original	190.0	67.5		
"	New	152.5	82.0		
"	Original	151.0	83.0		
"	New + pyrogallol	141.5		73.5	
"	Original + pyrogallol	141.0		74.0	
Sheep, saturated with CO	New + hydrosulfite	108.0		7.5	72.5
" "	Original + hydrosulfite	108.5		8.5	70.5
		<i>P</i> = 0.2, <i>S</i> = 2.0, <i>A</i> = 0.5			
Human	New	142.0	53.0		
"	Original	143.5	54.0		
" saturated with CO	New + hydrosulfite	147.0		42.1	32.0
" "	Original + hydrosulfite	149.0		40.5	31.0

\* O<sub>2</sub> pressure determined through absorption.

succeeded in performing O<sub>2</sub> and CO<sub>2</sub> analyses in Van Slyke's volumetric microapparatus. Table I shows that our analyses agreed exactly with those performed with the original reagent.

The following example illustrates results obtained with the microapparatus in determining blood gases: in the large apparatus CO<sub>2</sub> 34.0 volumes per cent, O<sub>2</sub> 17.33 volumes per cent; in the

apparatus first described by Van Slyke in 1917 (5) CO<sub>2</sub> 36.55 volumes per cent, O<sub>2</sub> 18.1 volumes per cent.

#### SUMMARY

A modification of the reagent for use in the Van Slyke method of blood gas analysis is described; this reagent prevents protein precipitation and therefore permits more exact readings.

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## STUDIES IN GASTRIC SECRETION

### V. THE COMPOSITION OF GASTRIC JUICE AS A FUNCTION OF ITS ACIDITY\*

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Most of the investigators who have concerned themselves with the mechanism of formation of gastric hydrochloric acid have attacked the problem by attempting to devise a physicochemical system which is capable of producing this acid *in vitro* and in the initial absence of any strong acid. Such a system, however, in order to be applicable in the construction of a cell model, must account quantitatively for at least the major constituents of the parietal secretion. At present we have little information concerning the nature of this fluid, for no way has yet been discovered whereby it can be obtained in the pure state. However, a previous study of this series suggested that some notion of the chemical composition of the parietal fluid might be obtained from an examination of gastric pouch juice, if the characteristics of the latter were studied as a function of the acidity of the mixed secretion. By a process of extrapolation, one might arrive at the magnitudes of these characteristics for pure parietal secretion with a high degree of probability.

Although the literature contains many studies which involve two or three properties of gastric juice, only three extensive analyses have ever been reported. These are contained in the well known papers by Rosemann (1907) on gastric juice from dogs obtained by sham feeding, by Carlson (1915) on appetite juice from human beings, and by Gamble and McIver (1928) on gastric juice from cats provided with Heidenhain pouches. On examining these

\* A preliminary report of this work was presented in *J. Biol. Chem.*, 97, xli (1932); *Proc. Am. Soc. Biol. Chem.*, 8, xli (1932).



sets of data, it was found that none of them was suitable for the present purpose, either because the number of samples reported was not sufficiently large or else because there were no determinations of acidity adequate for the desired correlation. On the other hand, with the animal technique used in this laboratory it is possible to obtain gastric pouch juice in quantity and entirely free from the various contaminants which are so frequently present. Consequently, in the present work, extensive analyses have been carried out on a series of large samples of pure gastric juice of different acidities. The results have been correlated with the total acidity values, and it has thus been possible to infer a number of the quantitative characteristics of the pure acid-containing secretion as it is elaborated by the parietal cells.

### *Methods*

Pure gastric juice was collected under a variety of conditions from several dogs with fundic pouches. All of the precautions to prevent contamination were observed as usual. Each sample so collected was filtered immediately to remove undissolved mucin and the total acidity was determined. The samples were arranged in order of increasing acid concentration, they were then divided into five groups, the component samples of each of which possessed nearly the same acidity. On mixing, there resulted five large composite specimens of increasing acid concentration. Only a very small proportion of all the samples collected had acidities of less than 100 mM and these possessed volumes so small as to prevent the preparation of a composite specimen of this order of acidity. Accordingly, such samples were included in the mixture of lowest acidity. It so happened that one of the dogs (Dog Y) was in particularly good condition for the collection of juice of constant acidity. Consequently, Specimens 4 and 5 are made up almost entirely of material from this animal.

Total and free acidities were determined by titration to definite pH end-points as previously described (Hollander, 1931); total chlorine by the method of Wilson and Ball (1928); phosphorus by the method of Fiske and Subbarow (1925), 2 cc. samples of gastric juice being used. Specific gravity determinations were performed with the Westphal balance; the results were recalculated with reference to water at 4°. Freezing point depressions were de-

terminated with all the usual precautions; the values as given are corrected for exposed stem and supercooling.

Total solids (dry weights) were determined as follows: 25 cc. portions of gastric juice were pipetted into silica beakers and evaporated down just to dryness in an electric oven at 100–105°. To minimize charring, the beakers were supported on glass shelves instead of on metal ones; also, within 30 minutes after they became dry they were transferred to a desiccator for cooling and a first weighing. A second weighing was made after 2 hours of additional heating. In no case did the two results, expressed as *per cent of total solids*, differ from each other by more than 0.01. Duplicate determinations, completely independent, also agreed to within these limits; the weight finally accepted was the mean of these four results.

To determine inorganic solids, the organic matter was destroyed by a wet combustion method. Following the determination of total solids, 2 cc. of concentrated nitric acid were added to each silica beaker and the latter was heated gently over a very low flame. 1 or 2 drops of 30 per cent hydrogen peroxide were then added with twirling of the beaker and occasional addition of heat. This was repeated at 3 to 5 minute intervals until a total of 10 drops of reagent had been added. The beaker was kept covered with a watch-glass whenever possible in order to reduce evaporation. Following the addition of the last drop, the contents were evaporated almost to dryness and the entire procedure repeated; a third treatment was found unnecessary. After the second evaporation the residue was converted to chloride by adding 2 cc. of concentrated hydrochloric acid, heating gently for 20 to 30 minutes, and finally evaporating. This treatment also was repeated, after which the beaker was dried in the oven and weighed as usual. Blanks were run on all the reagents. Results were calculated as gm. of ash (as chloride) per 100 cc. of gastric juice.

In order to obtain some kind of check on this method, the results were compared with the corresponding neutral chloride values by recalculating the latter to gm. per 100 cc. To do this it was assumed that the fixed base is present essentially as an equimolecular mixture of sodium and potassium. This gives an average molecular weight (as chloride) of 66.5 to be used in the calculation. In this way the value of per cent ash minus per

cent neutral chloride was calculated for each specimen. The resulting differences were 0.03, 0.02, 0.00, 0.04, and 0.05 per cent respectively; their average is 0.028 per cent. Further, it was desired to compare this wet ashing method with the dry method used by Gamble and McIver (1928), *i.e.* that of Stolte (1911). The average value for per cent ash minus per cent fixed base chloride (determined from the weights of sodium, potassium, and calcium) was calculated for the three specimens of Gamble and McIver's Table I. The results were  $-0.067$ ,  $-0.053$ , and  $-0.074$  per cent respectively. The average difference is  $-0.065$  per cent. It may be concluded, therefore, that the procedure used in the present investigation yields better results than does the ignition method.

Throughout this work all determinations were made in duplicate.

*Observations*—The data for all five specimens of juice are presented in Table I. As the total acidity increases from 111 to 158 mm, there is a progressive decrease in the concentrations of all constituents other than hydrochloric acid. Combined acidity, calculated as the difference between free and total acidities, decreases from 4 to 2 mm; the latter value is negligible within the limits of error of the method employed. Neutral chloride, calculated as the difference between total chloride and total acidity, decreases from 28 to 5 mm. Although the reliability of these values is probably no better than 1.5 mm,<sup>1</sup> and may be even worse, the lowest value is not quite small enough to indicate the complete absence of neutral chloride. Neither inorganic phosphate nor organic phosphorus could be detected in the two specimens of highest acidity. Phosphorus determinations on the other three specimens were therefore not considered necessary for our present purpose. As the acidity rises, the content of organic substances and the content of ash both decrease, the former from 0.15 to 0.04 per cent, the latter from 0.20 to 0.08 per cent. The minimum values, for Specimen 5, are both small, but neither of them is low enough to be considered negligible. The progressive fall in organic matter with increasing acidity is supported by the results with the biuret and Hopkins-Cole reactions. In both cases the intensity of coloration decreases with rising acidity until the test becomes

<sup>1</sup> In an earlier consideration of this point this value was misprinted as per cent instead of mm (Hollander, 1932, p. 588).

negative. The Molisch test, with and without previous heating, was negative in all instances. The specific gravity ( $D_{22/4}$ ) value is 1.001 for all specimens except that of lowest acidity, for which it is 1.002. The variation of 0.001 actually results from a variation of only 0.0003 or 0.0004, a difference which is probably related to the progressive increase in total solids. These values are in excellent agreement with those calculated for pure hydrochloric acid from

TABLE I  
*Some Chemical Characteristics of Gastric Juice*

	Specimen 1	Specimen 2	Specimen 3	Specimen 4	Specimen 5	Parietal secretion*
Total acidity, <i>mM</i> .....	111	136	144	155	158	170 <i>ca.</i>
Free " ".....	107	133	141	153	156	170 "
Combined acidity, <i>mM</i> .....	4	3	3	2	2	None
Total chloride, ".....	139	158	162	162	163	170 <i>ca.</i>
Neutral chloride, ".....	28	22	18	7	5	None
Organic P.....				0	0	"
Inorganic P.....				0	0	"
Total solids, <i>per cent.</i> .....	0.35	0.24	0.20	0.13	0.12	"†
Ash as chloride, <i>per cent.</i> .....	0.20	0.17	0.12	0.09	0.08	"†
Organic solids, " ".....	0.15	0.08	0.08	0.04	0.04	"
Biuret test.....	++	+	+	++	±§	—
Hopkins-Cole test.....	++	+	+	++	—	—
Molisch test.....	—	—	—	—	—	—
Sp. gr. ( $D_{22/4}$ ).....	1.002	1.001	1.001	1.001	1.001	1.001
Freezing point depression, °C...				0.614	0.628	

\* The values in this column have been arrived at by extrapolation from the observed values.

† This is subject to the considerations pointed out on p. 40.

‡ Very faint.

§ So faint as to be uncertain.

standard data (International Critical Tables, 1928, *a*). For example, the value for specific gravity ( $D_{22/4}$ ) of 170 *mM* hydrochloric acid is 1.0013. The freezing point depressions of 0.614° and 0.628° for Specimens 4 and 5 were slightly greater than might be expected from the standard value (International Critical Tables, 1928, *b*) of 0.60° for pure hydrochloric acid (0.17 *N*<sub>w</sub>). This difference also can be explained by the presence of small quantities of solid substances in the gastric juice.

## DISCUSSION

Extensive studies of the total acidity and the total and neutral chloride concentrations of pure gastric juice (Hollander and Cowgill, 1931; Hollander, 1932) have already been reported. The general conclusions derived therefrom may be stated as follows: Gastric juice as ordinarily collected may possess any concentration of hydrochloric acid up to a maximum in the neighborhood of 170 mm. This upper limit corresponds to the pure parietal secretion, free from mucus, peptic secretion, etc.; it is these other fluids which normally cause a reduction from the maximum acidity by dilution and neutralization. The higher the acidity, the lower the neutral chloride concentration; the limiting value for the latter is zero. In agreement with this, the concentration of total chloride in gastric juice also approaches a maximum value of about 170 mm.

Although it is common practice in studying the various chemical constituents of gastric juice to correlate their concentrations either with the rate of flow or with the time interval between stimulation and collection of the secretion, a different method was used in the study of total and neutral chlorides. Since it had previously been found that neither the rate nor the time interval is primarily related to the acidity, the concentration of chloride was plotted against the total concentration of acid. This method for analyzing the data having proved fruitful in its previous application, the same procedure has been followed in the present investigation. Thus, graphs of the present data plotted against the total acidity have been extrapolated to an acidity of about 170 mm,<sup>2</sup> with the results shown in the last column of Table I.

The combined acid values are all very low and they decrease progressively with increasing total acidity to an extrapolated value of less than 2 mm. This result is supported by the observations of Fouts *et al.* (1933) that "the higher the total acid, the less combined acid is present." Their values for human gastric juice are

<sup>2</sup> It must be remembered that a hydrochloric acid solution of this concentration possesses about the same osmotic pressure as dog blood does ordinarily. Deviations from normal values in the latter will cause corresponding changes in the acidity of the parietal secretion. The use of 170 mm for extrapolation, therefore, constitutes a reference value on the assumption that the animals employed in the investigation possessed blood of normal or average osmotic pressure.

also as low as 2 and 3 mm. Since titration of pure hydrochloric acid may also yield a combined acidity value of 1 to 2 mm, in conformity with the titration curve for this acid and the experimental errors involved, it may be concluded that pure parietal secretion contains no combined acid.

In the case of total and neutral chloride concentrations, the results obtained here on five large mixed specimens of gastric juice confirmed those previously reported for a large number of individually collected samples; namely, with increasing acidity the neutral chloride value approaches zero. In fact, some of the individual samples of gastric juice that entered into Specimen 5 possessed neutral chloride values as low as 3 mm; this value is almost negligible within the limits of error of the several experimental procedures involved in its determination. Thus there can be no doubt that the chlorine content of gastric juice of the highest degree of purity conceivable, *i.e.* parietal secretion, is entirely in the form of hydrochloric acid and not at all as alkali or alkaline earth chloride.

Phosphorus determinations were performed only on the two specimens of highest acidity (Specimens 4 and 5). The fact that no organic or inorganic phosphorus could be detected in either case would seem to indicate that the parietal secretion likewise is phosphorus-free. Although this result has never before been reported, it has been suspected by other investigators. Of the early workers, von Tabora (1905) declared phosphate to be of no importance in gastric juice; Rosemann (1907) could find no inorganic phosphorus in gastric juice from dogs, but reported a total phosphorus content as low as 0.27 mg. per 100 cc. More recently, Gamble and McIver (1928) obtained values as low as 0.16 mg. per 100 cc.; study of the results in their Table I shows that the higher the total chloride concentration, the lower is the phosphorus value. Similarly, Helmer, Fouts, and Zerfas (1932) found that the total phosphorus content of human gastric juice decreases progressively as the total acidity increases. Although they did not obtain exceedingly high acidities, their phosphorus values were as low as 0.6 mg. per 100 cc. at an acidity of 135 mm (Fouts, 1933). Thus, it may be concluded that inorganic phosphate and organic phosphorus in gastric juice are derived entirely from the mucous secretion and other contaminants of the parietal fluid, but not at all from the latter itself.

The results of the dry weight determinations are in harmony with the other observations. With increasing acidity, the value for total solids decreases progressively to a minimum of 0.12 per cent. The corresponding minimum values for inorganic and organic solids are 0.08 and 0.04 per cent respectively. For comparison with these, we have the following minimum values for total, inorganic, and organic solids respectively, found by other investigators: Rosemann (1907) 0.26, 0.08, 0.18 per cent; Carlson (1915) 0.48, 0.11, 0.34 per cent; Gamble and McIver (1928) 0.17, 0.12, 0.05 per cent. The data of Rosemann and of Carlson are not in a form for correlation with the acid concentrations; the values of Gamble and McIver, however, all decrease with increasing concentration of total chlorine and therefore with acidity (which was not determined in their experiments). Our findings for organic solids are also supported by those of Webster (1930) that in any one collection experiment, the minimum value for dissolved mucin (precipitated by adjustment of the pH) corresponds to the maximum acidity attained during that experiment. In fact, the curve for mucin content closely parallels the curve for neutral chloride concentration. Finally, the unmistakable gradation in intensity obtained in our own work with the biuret and Hopkins-Cole tests, both of which became negative in the sample of highest acidity, furnishes additional evidence in support of the generalization that the higher the acidity of these samples of pure gastric juice, the lower is their content of both organic and inorganic solids.

In order to determine the limiting values of these variables, corresponding to the acidity of pure parietal secretion, they were both plotted against the total acidity. In the case of the graph for organic solids, the individual points are well distributed about a straight line which passes through zero at an acidity around 170 mm. For inorganic solids, the fit to a straight line is good except for Specimen 1, which deviates from it appreciably. This line also approaches a zero value for inorganic solids, but at an acidity of 180 mm; the value for inorganic solids at 170 mm is about 0.03 per cent. Consequently, it cannot be said with certainty from these data alone that the parietal fluid is entirely free of inorganic solids. Nevertheless, in consideration of the fact that both phos-

phate and neutral chloride are absent from this secretion, it seems very probable that the same is true for inorganic solids as well.

The specific gravity values varied so little from each other—within the fourth decimal place only—that the value 1.001 may be accepted as the specific gravity of the parietal secretion. This figure agrees with that for pure hydrochloric acid of 170 mm concentration. The freezing point depression values of  $0.614^{\circ}$  and  $0.628^{\circ}$  are sufficiently close to the figures reported for dog blood to leave no doubt as to their significance. This finding is well supported by the conclusion arrived at in the very thorough study of this phase of the problem by Gilman and Cowgill (1933), that blood and gastric juice are practically isotonic, with the osmotic pressure of the gastric juice almost entirely a function of its chloride concentration. Changes in the blood values which they induced experimentally were paralleled by changes in the gastric juice values. Thus, both the specific gravity and the freezing point depression of the parietal secretion correspond to those of a hydrochloric acid solution which is isotonic with the blood; *i.e.*, about 170 mm.

#### SUMMARY

In the foregoing study much additional evidence has been adduced in support of the hypothesis, previously formulated, that "The parietal secretion is essentially an isotonic solution of hydrochloric acid. . . ." It has been shown not only that this secretion contains no neutral chloride, but that combined acid, inorganic phosphate, organic phosphorus, and organic solids are all absent. Inorganic solids are probably also absent, though this has not been established conclusively. The specific gravity conforms to that of a hydrochloric acid solution of 170 mm concentration; the freezing point depression is such as to establish the isotonicity of the pure secretion. In short, if any substances other than hydrochloric acid and water are contained in the parietal secretion, their quantities are so small as to be negligible in the initial formulation of any hypothesis concerning the formation of this fluid. Having thus established the essential characteristics of the parietal secretion, it should now be possible to proceed with a study of the thermodynamics of its formation—and thereafter, perhaps, of its mechanics.



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# THE ULTRAFILTRATION OF MALT AMYLASE SOLUTIONS

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## INTRODUCTION

Semipermeable membranes have been used for the study of diffusion phenomena since 1855 (1). Several methods of securing membranes having regular gradations have been employed, in order to control the permeability with respect to certain solutes. In one method the concentration of nitrocellulose in the collodion is varied (2). The time of evaporation of the solvent may be varied regularly (3), or evaporation may be allowed to proceed until the membrane reaches a definite weight (4). The ratio of alcohol to ether in the collodion may be varied (5). Different amounts of substances other than alcohol and ether, such as glycerol (6), ethylene glycol (7), or ethyl formate or ethyl acetate (8), may be used in preparing the collodion. Air-dried membranes may be graded by immersion for a definite time in varying concentrations of alcohol before transferring them to water (9). These methods are all directed toward the same end; that is, a control of the ratio of swelling agent to nitrocellulose in the membrane at the time of immersion in water. Increasing amounts of swelling agent present at the moment of immersion in water cause increased imbibition of water by the membranes and greater permeability. By the above methods of preparation it has been shown that the permeability of a membrane varies directly with the amount of swelling agent present either in the collodion (5, 7) or in the first washing liquid (9). It varies inversely with the concentration of nitrocellulose used (2), and with the extent of evaporation of the solvent (3, 4).

The usefulness of graded membranes lies in the fact that they permit fractional dialysis. Certain solutes pass through while

others do not. The behavior of a particular substance depends on the grade of membrane used. In the work described in this paper graded nitrocellulose membranes were made in order to study quantitatively their permeability to malt amylase as well as some of the factors affecting this. The method of grading chosen was the introduction of varying amounts of ethylene glycol (7) into the collodion.

*Preparation of Membranes*—The method is essentially that of Pierce (7), with a somewhat shortened procedure. Throughout this work the concentrations of nitrocellulose and of alcohol in the collodion were kept constant. Varying amounts of ethylene glycol were added to 25 cc. of absolute alcohol and the whole diluted to 100 cc. with anhydrous ether. The 100 cc. of solvent were poured onto 2 gm. of nitrocellulose, which had been air-dried, and then stored in a desiccator in a refrigerator. After a gradual swelling process over a period of several hours, the nitrocellulose finally goes into solution on shaking.

The per cent by volume of ethylene glycol in the solvent is used to designate the *grade* of the resultant membrane. A membrane made from collodion containing 4 cc. of ethylene glycol in 100 cc. of solvent is called a membrane of Grade 4.

In making membranes, 6 cc. of collodion were pipetted onto a polished glass plate, 9 cm. in diameter. The collodion spread out in a smooth film, covering the entire surface of the plate. The glass plates were precisely ground so that both faces were flat and parallel. Four membranes were prepared at a time. The evaporation chamber and other equipment were those used by Field (10). Air was aspirated through the chamber for a period of 6 hours at the rate of  $50 \pm 1$  cc. per minute. The air passed through 8 mesh granules of anhydrous calcium chloride at the entrance and exit to the chamber. Fresh calcium chloride was used each time at the exit. The temperature was that of the room; as a rule it varied from 22–25°.

At the end of the 6 hour evaporation period the chamber was opened, one membrane was covered immediately with a watch-glass, and the other three were transferred on their plates to distilled water at 22–25°. The covered membrane was weighed as quickly as possible to get the weight of nitrocellulose plus organic solvent present. This is designated as the *organoweight*. The

membrane was then immersed in water with the others. When the membranes had floated free from the plates, they were put in individual dishes of distilled water and allowed to stand overnight.

After washing, the wet weight was obtained by weighing a membrane in a covered weighing bottle after blotting off the surface moisture with filter paper. The dry weight was obtained by heating to constant weight. The membranes were stored under water, in which manner they may be kept for several weeks without alteration in properties.

TABLE I  
*Effect of Ethylene Glycol on Weights of Membranes*

These are average values, given in mg.

Membrane Grade* No.	Dry weight	Organoweight	Wet weight	$G_o$ †	$G_w$ ‡	$R$ §
0	114	135	124	0.18	0.09	2.10
4	112	630	402	4.63	2.59	1.79
6	113	760	520	5.72	3.60	1.59
8	112	935	625	7.35	4.58	1.60
10	112	1090	750	8.73	5.70	1.53
12	112	1280	886	10.43	6.91	1.51
14	112	1427	1024	11.74	8.14	1.44
16	112	1583	1156	13.13	9.32	1.41
18	112	1751	1237	14.63	10.04	1.46
20	112	1801	1298	15.08	10.59	1.42

\* Grade = per cent by volume of ethylene glycol in the solvent used in making collodion.

†  $G_o$  = (organoweight - dry weight)/dry weight.

‡  $G_w$  = (wet weight - dry weight)/dry weight.

§  $R$  = (organoweight - dry weight)/(wet weight - dry weight).

The relatively high wet weights and corresponding permeability of the membranes prepared under the conditions described are due to the presence of ethylene glycol in the collodion. Since the vapor pressure of the ethylene glycol is negligible at room temperature (11), it remains in contact with the nitrocellulose during the evaporation period. It retains with it a part of the alcohol (10). Increasing amounts of ethylene glycol in the collodion resulted in membranes showing a corresponding increase in weight, as shown in Table I.

*Reproducibility of Membranes*—A series of membranes including Grades 0 to 20 were prepared over a period of 18 months. During the warmer months difficulty was experienced in obtaining membranes whose values checked with those made previously. This was found to be due to high atmospheric humidity, which caused an increase in wet weight. Membranes can be reproduced with reasonably good checks during periods of relatively low humidity. The mean of the average deviations for membranes of the same grade is  $\pm 2.6$  per cent, which may be used to express the average reproducibility of the membranes under the conditions described.

### *Ultrafiltration of Malt Extract*

*Preparation of Extract*—The extract was made by adding 4 parts of cold water to 1 part by weight of ground malt barley. The mixture was shaken by mechanical rotation for 1 hour, decanted into a centrifuge cup, centrifuged for 10 minutes, and filtered in a refrigerator. A slightly turbid brown liquid was obtained. The extract was stored on ice overnight and used the next day for ultrafiltration.

Before use the extract was buffered to pH 4.3 to 4.5, the isoelectric zone of malt amylase (12), with an acetic acid-sodium acetate mixture (13, 14). The concentration of buffer needed to overcome the buffering action of the proteins present was determined by titrating the extract electrometrically with 0.1 M acetic acid. The proportion of the two buffer constituents to be used was determined by titrating an extract made 0.03 M with acetic acid against that made 0.03 M with sodium acetate.

Ultrafiltration was selected rather than dialysis, as it was planned to test quantitatively the liquid on each side of the membrane for diastatic activity. A dilute solution of enzyme material is less stable than a concentrated solution, which is attributed to hydrolysis (15). This suggests that in ordinary dialysis negative tests for active enzyme in the diffusate would be inconclusive, as the volume of diffusate is usually large with respect to the volume of the dialyzed solution. In ultrafiltration the total volume of solution is unchanged.

The ultrafilter was similar to that designed by Pierce (7). 40 cc. of extract containing a 0.03 M acetate buffer were put in the ultrafilter, the latter adjusted in a shaking machine, and a pressure

of 300 mm. of mercury applied by connecting the ultrafilter to a nitrogen tank and manometer. 30 cc. of filtrate, three-fourths of the volume of extract used, were caught in six successive 5 cc. portions. Enzyme action was determined by the gravimetric method of Sherman, Kendall, and Clark and calculated according to their scale (16). The starch solution was buffered to a pH of 4.5 (17) with 0.01 M acetate (14). Ordinarily enzyme action was determined on 0.04 cc. of the original extract, 0.5 cc. of each of the six filtrates, and 0.02 cc. of the residue. By using an extract prepared fresh each time under standard conditions, the diastatic power did not differ greatly from one time to another. The mean diastatic power for twelve extracts prepared as described was 38.1, with an average deviation of  $\pm 1.07$ , or  $\pm 2.8$  per cent. Total solids were determined by evaporating to dryness 2 cc. of each of the eight fractions, at  $70^\circ \pm 2^\circ$ . Determinations of hydrogen ion activity on the original, the filtrates, and the residue were all within the limits pH 4.3 to 4.5.

*Results with Malt Extract*—The filtrates were always clear and only slightly colored; the residue was always turbid and contained a precipitate which settled out. Qualitative color tests were made on several occasions. In each case all six portions of the filtrate gave positive Molisch and positive xanthoproteic tests. In each case the original extract and the residue gave positive biuret tests. Filtrates which did not contain active enzyme in some cases gave a doubtful biuret test; that is, a blue rather than a violet color. The filtrates were not concentrated for these tests.

The time required to collect each successive 5 cc. portion of filtrate increased progressively. The solid content of twelve samples of extract prepared under like conditions was  $42.8 \pm 1.0$  mg. per cc. The average deviation is  $\pm 2.3$  per cent. The filtrates showed a gradual increase in total solids with the volume of liquid filtered, as shown by the following data, representing total solids in mg. per cc.

Membrane Grade No.	Original extract	Filtrate No.						Residue
		I	II	III	IV	V	VI	
8	42.4	34.2	36.2	36.2	36.9	37.2	37.5	54.2
10	42.2	35.1	36.7	36.7	37.4	37.6	38.6	47.5
12	41.7	34.0	36.9	36.9	37.6	38.0	39.1	44.0

When the original extracts are very similar in their solid content, differences in the permeability of membranes of different grades can be demonstrated by the amount of material passing through them. Increasing amounts of dissolved substances were carried through the membranes as the grade increased, except in the case of Filtrate I. Confirmatory evidence of the increase in permeability is furnished by the amount of material left in the residue, which decreased as the grade of membrane increased.

Membranes up to and including Grade 8 were impermeable to the enzyme under the conditions described. Membranes of Grade 9 and over were permeable. With a membrane permeable to the enzyme a sudden increase in activity was observed in Filtrate V or VI. In all cases Filtrates I to IV yielded in the neighborhood of 50 mg. of cuprous oxide per 0.5 cc. This was the actual blank for the filtrate. This was shown by inactivating the enzyme by boiling samples before the enzyme determinations were made. The presence of reducing substances in the filtrates is evident, as a blank on the reagents used ordinarily yielded 37 mg. of cuprous oxide.

A rough estimate of the recovery of enzyme activity may be made by calculating the original activity of the 40 cc. of extract put in the ultrafilter and comparing with the total activity of the filtrates plus that of the residue, assuming the latter to be 10 cc. The result is as follows:

Membrane Grade No.....	8	8	9	10	12
Per cent recovery.....	98	70	55	47	39

With Grade 8 membranes, variable results were obtained in calculating the recovery of total potency. This is probably because these membranes were near the border line of permeability under the conditions described. As more of the enzyme passes through a membrane, more of it is lost. This indicates that malt amylase, like protein material (18), is adsorbed by nitrocellulose membranes. The amount of enzyme removed in passing through a membrane may be expected to increase as the membrane thickness becomes greater. As the membrane grade decreases and the nitrocellulose gel becomes more compact, a point may be reached where the size of the pores becomes too small to permit the entrance of enzyme material (19).

By the use of relatively impermeable membranes enzyme potency was increased by ultrafiltration, in terms of solid content, to about 3 times that of the original extract.

*Adsorption Tests*—To test the validity of the conclusion that a membrane is able to adsorb active enzyme, experiments were made with a partially purified product obtained by precipitation of a protein fraction with 65 per cent alcohol (15). 20 mg. of the dried product were rubbed up with water, buffered to give a pH value of 4.4, and diluted to 50 cc. One portion of enzyme solution was kept as a blank, a second portion was placed in contact with a membrane, and a third portion was placed in contact with a membrane which had been previously soaked in a 0.1 per cent solution

TABLE II  
*Adsorption of Enzyme by Membranes*

The enzyme action is expressed as mg. of  $\text{Cu}_2\text{O}$  per cc. of solution.

	Test No. (1)	Action after 1 day (2)	Per cent loss (3)	Action after 4 days (4)	Per cent loss (5)
Control enzyme solution	1	355		355	6
	2	394		384	3
Enzyme solution in contact with untreated membrane	1	120	66	26	92
	2	186	53	37	90
Enzyme solution in contact with membrane treated with albumin solution	1	214	40	104	69
	2	263	33	130	66

of egg albumin. The three solutions were tested for diastatic power after standing for 1 day and for 4 days in a refrigerator, with occasional shaking. Two such experiments were made. Grade 12 membranes were used. The results are given in Table II.

In Column 3 of Table II is given the per cent loss of potency of the enzyme solution in contact with both untreated and treated membranes, calculated from the potency of the 1 day-old control enzyme solutions. In Column 5 the first two figures give the loss of potency of the control enzyme solutions after standing 3 days longer than in the first determinations. The succeeding figures in Column 5 give the per cent loss of potency based on the 4 day control.

The tubes were shaken vigorously immediately before removing



samples for testing. As may be seen from Table II, a large proportion of the enzyme potency is lost by contact with a membrane. This loss is significantly less if the membrane has first been soaked in a solution of egg albumin. From this it is concluded that the enzyme is adsorbed by a nitrocellulose membrane in the same way that egg albumin is adsorbed.

#### SUMMARY

1. Nitrocellulose membranes graded by means of ethylene glycol have organoweights and wet weights which are proportional to the amount of ethylene glycol in the collodion.

2. The membranes are ready for use in one-third the time required by the procedure of Pierce.

3. That the permeability of a membrane corresponds to its grade may be demonstrated by a determination of total solids on the filtrates obtained from the ultrafiltration of malt extract.

4. Malt extract may be purified 3-fold in terms of solid content by ultrafiltration, by the use of membranes impermeable to the enzyme but permeable to more highly dispersed substances.

5. Enzyme material is adsorbed by nitrocellulose membranes in the same way that egg albumin is adsorbed.

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# **A COMPARISON OF THE METHODS FOR THE COLLECTION OF BLOOD TO BE USED IN THE DETERMINATION OF GASES**

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For the collection of blood for the determination of the gaseous content we have made use of a device by which the samples can be taken and stored in ordinary glass syringes. The syringes were carefully selected so that the plunger fitted the barrel very closely. In order to prevent the blood from seeping between the plunger and barrel and causing them to stick, a very small amount of white petrolatum was applied to the sides of the plunger. Care was taken that the petrolatum did not coat the end of the plunger and thus interfere with the solution of the anticoagulant. This consisted of a mixture of sodium fluoride and potassium oxalate which was ground to an impalpable powder and placed inside the barrel of the syringe so as to fill the depression made by the junction of the nipple and barrel, and the plunger was forced home so as to expel all the air. The blood was drawn with a minimum of suction and the syringe immediately closed with a cap made by fusing the end of a hypodermic needle from which the shank had been broken off. The syringe was then rapidly rolled between the hands to insure complete mixing of the anticoagulant with the blood. The amount of air remaining in the syringe is so small in comparison with the total amount of blood drawn as to introduce no appreciable error, but it is preferable to eject this before applying the cap.

In order to determine the changes which occur when oil is used, duplicate analyses were made by the Van Slyke manometric technique on twenty-two blood samples collected by this method, drawn directly into a tube containing oil, or drawn in an oiled

TABLE I  
*Comparison of Methods for Analyses of Venous Whole Blood*

	No. of cases	Mini- mum	Maxi- mum	Mean		Standard deviation	Correlation coefficient Analysis 1 versus Analysis 2	Approximate time between drawing and analysis of blood hrs.
		vol. per cent	vol. per cent	vol. per cent	vol. per cent			
CO <sub>2</sub>								
Syringe method								
Analysis 1.....	22	49.05	62.22	56.795 ± 0.312	1.430 ± 0.218	0.981 ± 0.008	2-3	
“ 2.....	22	49.80	62.87	56.575 ± 0.318	1.459 ± 0.222			
Transfer from syringe to tube containing oil								
Analysis 1.....	19	49.00	62.60	54.782 ± 0.330	1.399 ± 0.230	0.891 ± 0.049	1-2	
“ 2.....	19	50.33	62.14	55.113 ± 0.303	1.285 ± 0.211			
Direct suction into tube containing oil								
Analysis 1.....	18	48.75	58.65	54.525 ± 0.246	1.014 ± 0.171	0.912 ± 0.041	1-2	
“ 2.....	18	50.34	56.36	54.370 ± 0.202	0.808 ± 0.141			
O <sub>2</sub>								
Syringe method								
Analysis 1.....	22	6.01	18.60	11.499 ± 0.341	1.564 ± 0.239	0.997 ± 0.001	2-3	
“ 2.....	22	6.61	19.27	11.698 ± 0.348	1.596 ± 0.243			
Transfer from syringe to tube containing oil								
Analysis 1.....	19	7.76	20.61	13.172 ± 0.392	1.665 ± 0.274	0.972 ± 0.013	1-2	
“ 2.....	19	5.70	18.72	12.491 ± 0.354	1.504 ± 0.247			
Direct suction into tube containing oil								
Analysis 1.....	18	6.41	18.98	13.022 ± 0.368	1.517 ± 0.256	0.976 ± 0.012	1-2	
“ 2.....	18	6.55	18.61	12.419 ± 0.322	1.288 ± 0.224			

syringe and then transferred to a tube containing oil. The latter samples, containing 10 ml. of blood, were kept in  $150 \times 18$  mm. test-tubes, in which they formed a layer 5 cm. deep, and were covered by a layer of oil also 5 cm. in depth. The analyses of the duplicate samples kept under oil were always made before those stored in the syringe. The blood stood under oil from  $\frac{1}{2}$  to 2 hours before the analyses were completed, but was held in the syringes from 2 to 3 hours.

The period of time which should elapse in order to allow the circulation again to reach normal, and to avoid the effect of stasis caused by the application of the tourniquet, was also studied. In general usage 30 seconds are assumed to allow sufficient time for equilibrium to be established after removal of the tourniquet. Duplicate analyses were made on bloods collected 30, 90, and 150 seconds after the tourniquet was removed. All samples were taken under basal conditions from male schizophrenic patients.

In order to conserve space, only a summary of the results of the analyses, comparing the effects of differences in method, is given in Table I. It will be noted that the selfcorrelation between the first and second analyses is much higher for the syringe method than for the oil methods. This indicates that the precision of the method is affected by the oil, which prevents the thorough mixing of the specimen so as to obtain homogeneous samples.

The mean of the differences between the carbon dioxide content of blood stored in the syringes and that kept under oil was  $+1.85$  volumes per cent for the transfer method and  $+1.88$  volumes per cent for the direct suction method. The maximum difference obtained in the series was  $+7.98$  volumes per cent, while differences of 2.00 volumes per cent or more occur in half of the analyses. When analyzed by "Student's" method<sup>1</sup> for the significance of differences between small samples, it was found that the differences between the syringe method and the oil methods were highly significant, as the odds against their being due to chance were 9999 to 1 and 908 to 1, but that there was no significance for the difference between the two oil methods.

The mean of the differences for the oxygen content was  $-1.28$  volumes per cent between the syringe and the transfer method and  $-1.37$  between the syringe and the direct suction method.

<sup>1</sup> "Student," *Biometrika*, 6, 19 (1908).

TABLE II  
Effect of Stasis on Gaseous Content of Venous Whole Blood

	No. of cases	Minimum	Maximum	Mean		Standard deviation		Correlation coefficient Analysis 1 versus Analysis 2
		vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	
CO <sub>2</sub> 2½ min.								
	Analysis 1.....	20	47.30	61.06	55.291 ± 0.404	1.761 ± 0.282	0.985 ± 0.007	
	“ 2.....	20	47.33	61.02	55.054 ± 0.388	1.648 ± 0.271		
	1½ min.							
“ 1½ min.								
	Analysis 1.....	22	41.82	62.43	55.393 ± 0.533	2.442 ± 0.372	0.994 ± 0.003	
	“ 2.....	22	41.96	61.67	55.152 ± 0.519	2.379 ± 0.363		
	½ min.							
“ ½ min.								
	Analysis 1.....	22	42.70	64.82	56.115 ± 0.558	2.557 ± 0.390	0.997 ± 0.001	
	“ 2.....	22	42.82	64.56	56.021 ± 0.547	2.505 ± 0.382		
	O <sub>2</sub> 2½ min.							
“ 2½ min.								
	Analysis 1.....	20	7.37	19.75	13.416 ± 0.314	1.370 ± 0.219	0.996 ± 0.002	
	“ 2.....	20	8.64	19.95	13.534 ± 0.307	1.303 ± 0.214		
	1½ min.							
“ 1½ min.								
	Analysis 1.....	22	7.30	20.35	12.723 ± 0.343	1.574 ± 0.240	0.999 ± 0.0004	
	“ 2.....	22	7.02	19.94	12.710 ± 0.339	1.551 ± 0.237		
	½ min.							
“ ½ min.								
	Analysis 1.....	22	5.49	19.86	12.139 ± 0.369	1.693 ± 0.258	0.997 ± 0.001	
	“ 2.....	22	5.70	19.56	12.131 ± 0.364	1.670 ± 0.255		

The maximum increase in oxygen of the bloods stored under oil was 6.44 volumes per cent. The differences between the oxygen values for these methods are also significant, but considerably less so than for the carbon dioxide differences, as is shown by the odds, which were 181 to 1 and 62 to 1. This indicates that the oil is a more effective barrier for the passage of oxygen than it is for carbon dioxide.

The effect of stasis is shown in Table II, which summarizes the results of analyses of bloods taken at different time intervals. For this experiment all the bloods were taken by the syringe method. The reliability of this procedure is demonstrated by the high correlation coefficients, especially for the oxygen determinations, which, for the  $\frac{1}{2}$  minute interval, show a difference in the means of only 0.008 volume per cent.

There is no significant difference between the results obtained at the various time intervals except in the carbon dioxide figures for blood taken at  $\frac{1}{2}$  minute and  $1\frac{1}{2}$  minutes. The mean of the differences between these determinations is  $-0.775$  and the standard deviation of the differences is 1.171 volumes per cent, so that the odds against the differences being due to chance are 499 to 1. The mean of the differences for the carbon dioxide results at  $\frac{1}{2}$  and  $2\frac{1}{2}$  minutes is  $-1.124$  volumes per cent, but the standard deviation in this case is much higher, 2.566 volumes per cent, so that the difference is not statistically significant. The differences obtained for the oxygen values are not significant in any case, but the interval between  $\frac{1}{2}$  and  $1\frac{1}{2}$  minutes again showed the most marked difference. This tendency for the carbon dioxide to be higher and the oxygen to be lower in the half minute interval would indicate that there was some effect which could be ascribed to stasis. The interval which must elapse after the removal of the tourniquet should, therefore, be increased to 1 minute, at least in the case of schizophrenic patients.

#### SUMMARY

A method of collecting and handling blood for gas analyses in a capped syringe is described and is shown to prevent error from exchange of blood gases with the air.

Blood kept in 10 cc. portions for  $\frac{1}{2}$  to 2 hours under a layer of oil 5 cm. deep was found to lose  $\text{CO}_2$  and to absorb oxygen from the



air. The maximum loss in carbon dioxide was 7.98 volumes per cent; the mean loss, 1.86 volumes per cent. For oxygen the maximum gain was 6.44 volumes per cent, and the mean gain was 1.32 volumes per cent.

The effect of stasis on the gas content of blood can be avoided by allowing 1 minute to elapse after removing the tourniquet before drawing the blood.

## THE METABOLISM OF SULFUR

### XXI. COMPARATIVE STUDIES OF THE METABOLISM OF *l*-CYSTINE AND *dl*-METHIONINE IN THE RABBIT\*

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The extent of the oxidation of the sulfur of the amino acid, cystine, by the animal organism has been the subject of frequent study (1, 2). It has been shown that the sulfur of cystine administered in moderate quantities is oxidized readily and excreted in the urine chiefly as sulfate sulfur. Variable recoveries of the sulfur of cystine when fed are frequently obtained, owing presumably to the ready decomposition of cystine by the intestinal microflora with the formation of hydrogen sulfide and mercaptans. It has also been observed that, if the  $\alpha$ -amino group of cystine is blocked by some chemical group which prevents ready deamination in the organism (*i.e.* acetyl, benzoyl, etc.), the oxidation of the sulfur of the molecule is prevented to a very considerable extent (2-5).

Our knowledge of the conditions for the oxidation of methionine, the second amino acid containing sulfur present in the protein molecule, is not extensive. Mueller (6), who first isolated methionine as a product of the hydrolysis of protein, observed in a limited series of experiments a ready oxidation of the sulfur of small quantities of methionine when fed to man. Pirie (7), after administering *l*-methionine equivalent to 320 mg. of sulfur to dogs, was able to recover 79 to 86 per cent of the sulfur fed as "extra" sulfur in the urine. A similar ready oxidation of the sulfur of *dl*-methionine by the dog has been noted by White and Lewis (8) and by Stekol and Schmidt (9).

\* A preliminary report of this investigation was presented before the Twenty-seventh meeting of the American Society of Biological Chemists at Cincinnati, April 10-12, 1933 (*J. Biol. Chem.*, **100**, xciv (1933)).

The object of the present investigation was to afford a comparison of the oxidation of the sulfur of *l*-cystine and *dl*-methionine in the organism of the rabbit. The effect of blocking the  $\alpha$ -amino group of methionine by conjugation with the benzoyl group has also been studied.

#### EXPERIMENTAL

Male rabbits of 2 to 3 kilos weight were kept in the usual metabolism cages and fed uniformly 70 gm. of oats and 125 gm. of cabbage daily. This diet was eaten readily except on the days when the sulfur derivatives were administered, when part or, in some cases, all of the oats were occasionally not eaten. The urine was collected at regular intervals by gentle pressure on the abdominal wall. In some experiments, the samples of urine were collected in two portions (usually 6 and 18 hours) on the experimental days.

Total nitrogen of the urine was determined by the Kjeldahl-Gunning method, creatinine by Folin's micromethod, and the partition of urinary sulfur by the usual gravimetric procedures, the oxidizing reagent of Denis being employed in the determination of total sulfur. Disulfide linkages ( $-\text{SS}-$ ) were determined by a modification (10) of the iodometric method of Okuda and the results were calculated as cystine. Urines were examined for protein and thiosulfates by the usual qualitative tests.

The determination of the specific rotation of the cystine, prepared from hair in this laboratory, gave a value,  $[\alpha]_D^{20}$ , of  $-219.4^\circ$ . The methionine was a synthetic product. The *dl*-benzoylmethionine,<sup>1</sup> prepared according to the usual procedure for benzoylating amino acids, after purification melted at  $150^\circ$ , a melting point slightly higher than that reported for this compound by Windus and Marvel (11).  $\alpha$ -Phenylureidocystine was prepared by the method of Patten (12). All compounds administered showed satisfactory values for sulfur and nitrogen on analysis.

The sodium salts of the various compounds were usually administered. In the earlier experiments the hydrochlorides of cystine and methionine were used. However, the substituted derivatives of methionine and cystine, the behavior of which it was desired to investigate, do not form easily soluble salts with hydrochloric acid,

<sup>1</sup> We are indebted to Dr. Julius White, National Research Council Fellow in Medicine, for the preparation of this compound.

while the alkali salts of these compounds are sufficiently soluble to permit their use readily. Therefore in order to insure a uniform procedure, it was decided to administer the sodium salts of the various amino acids and their derivatives. Oral administration was accomplished by the use of a small rubber catheter introduced into the stomach and intravenous injections were made into the marginal ear vein. If more than one substance were administered to the same animal, intervals of at least 4 days between experiments were allowed in order to obtain satisfactory normal control values.

#### DISCUSSION

A summary of all the experimental data concerned with the elimination of "extra" urinary sulfur after the administration of the various compounds under investigation is presented in Table I. The results obtained with cystine are similar to the results recorded in the literature (1, 2) and indicate a variable recovery of the sulfur of the cystine administered as extra urinary sulfur, the greater part of the "extra" sulfur appearing as "extra" sulfate sulfur, *i.e.* in oxidized form, except after intravenous injection. When the  $\alpha$ -amino group of cystine was blocked by a group which prevented ready deamination as in  $\alpha$ -phenylureidocystine, little evidence of the oxidation of the sulfur was obtained, most of the extra urinary sulfur being excreted as organic sulfur, a finding in confirmation of the earlier work of one of us (3).

The amount of extra sulfur appearing in the urine after methionine was fed or injected was of approximately the same order of magnitude as after cystine, with one exception. In one experiment, in which methionine hydrochloride was fed, only 28 per cent of the sulfur of the compound was excreted in the urine. Apart from the probability of a very active destruction of the material fed by the intestinal microflora, no explanation for the low recovery of the sulfur in this experiment can be offered. The sulfur of the methionine molecule appeared to be as readily oxidized as the sulfur of the cystine molecule, when administered in equivalent amounts. Even when the methionine was introduced parenterally, a ready oxidation was observed. In one experiment only, in which methionine hydrochloride was injected intravenously, was the oxidation of the sulfur poor. In this experiment (Table I), 57

TABLE I

*Distribution of "Extra" Sulfur Excreted in Urine by the Rabbit after Administration of l-Cystine, dl-Methionine, and Their Derivatives*

In all the experiments, the amounts of the various compounds used were such that the equivalent of 400 mg of sulfur was administered. All the sulfur excretions are expressed as percentages of the total sulfur administered.

Compound	Mode of administration	Extra sulfur excreted		
		Total	Total sulfate	Organic
		per cent	per cent	per cent
Cystine hydrochloride	Oral	78	49	29
" "	"	62	25	37
" (sodium salt)	"	75	53	22
" " "	"	87	78	9
" " "	"	72	58	14
" " "	"	57	49	8
" " "	Intravenous	54	17	37
" " "	Subcutaneous	94	80	14
$\alpha$ -Phenylureidocystine (sodium salt)	Oral	61	11	50
" " "	"	54	10	44
" " "	Subcutaneous	61	-7	68
" " "	"	59	0	59
" " "	"	68	0	68
Methionine hydrochloride	Oral	85	57	28
" "	"	81	63	18
" "	"	59	35	24
" "	"	28	6	22
" "	Intravenous	70	46	24
" "	"	82	61	21
" "	"	66	38	28
" "	"	82	35	47
" (sodium salt)	Oral	61	32	29
" " "	"	89	64	25
" " "	Subcutaneous	67	40	27
" " "	"	54	54	0
" " "	"	67	51	16
" " "	"	82	72	10
$\alpha$ -Benzoylmethionine (sodium salt)	"	59	2	57
" " "	"	30	-3	33
" " "	Intravenous	55	11	44

per cent of the extra sulfur appeared in the urine as organic sulfur (82 per cent of the sulfur of the compound fed was excreted, 35 per

cent as inorganic sulfate sulfur and 47 as organic sulfur). This ready oxidation of the sulfur of methionine is comparable to that observed by Pirie in the dog (7).

The most interesting observation made in the series of experiments in which methionine was administered was the appearance uniformly in the urine of the experimental days of a substance which gave a positive cyanide-nitroprusside test. This test, which is commonly described as a test for cystine, is given by any substance which on reduction yields a sulfhydryl group. However, when the Sullivan reaction, a reaction more specific for the presence of cystine, was applied to these urines, negative results were always obtained. This is believed to indicate the excretion of some compound containing the disulfide grouping other than cystine.

Further evidence of the presence of such a compound was obtained from the quantitative data. The disulfide groups, as determined by a modified iodometric method showed a slight but unmistakable rise on the days when methionine was fed or injected. A typical experiment is presented in Table II. On the 5th day, the disulfide calculated as cystine which had ranged from 10 to 21 mg. on the preliminary control days increased to 43 mg. following the subcutaneous injection of methionine. Similarly on the 19th day, the excretion of disulfides calculated as cystine was 84 mg. following the oral administration of methionine. Similar evidence of increased excretion of disulfide compounds was obtained in every experiment with methionine. Slight increases in the excretion of disulfide were observed also after the administration of cystine (*cf.* the 24th day, Table II). That the increased values in these experiments were due to the presence of cystine which had escaped oxidation was shown by weakly positive Sullivan tests.

On heating with sulfuric acid, methionine has been found to yield a product which gives a positive cyanide-nitroprusside test, but no Sullivan test for cystine. From the products of the reaction, Butz and du Vigneaud (13) were able to isolate and identify the next higher homologue of cystine, to which the name, homocystine, was given. In our experiments, the reactions of the urine after the administration of methionine can be explained on the basis of the excretion of small amounts of homocystine, as a product of the intermediary metabolism of methionine. We have

prepared homocystine from methionine, according to the directions of Butz and du Vigneaud and have shown that, as anticipated, homocystine behaves similarly to cystine in the iodometric titration, that the disulfide group may be reduced, and the mercapto group thus formed may be determined by this method. De-

TABLE II

*Distribution of Urinary Sulfur after Administration of Methionine, Benzoylmethionine, and Cystine*

Rabbit J, weight 2.5 kilos. All the compounds were administered as the sodium salts in equivalent amounts (equivalent to 400 mg. of sulfur).

Day	Total N	Total S	Total sulfate S	Organic S	S-S linkages calculated as cystine	Day	Total N	Total S	Total sulfate S	Organic S	S-S linkages calculated as cystine
	mg.	mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.	mg.
1	1295	219	159	60	16	15	1180	224	150	74	23
2	1043	178	131	47	10	16	1131	236	154	82	41
3	1304	211	156	55	21	17	1292	210	159	51	10
4	1285	186	112	74	10	18	1460	209	161	48	21
5	1292	333*	277	56	43	19	1512	556†	401	155	84
6	1246	288	236	52	24	20	1091	177	120	57	15
7	1204	197	151	46	15	21	1343	235	177	58	8
8	1239	145	95	50	18	22	1414	237	172	65	39
9	1448	161	125	36	13	23	1551	186	138	48	10
10	1565	143	115	28	7	24	2045	492§	405	87	37
11	1352	238	177	61	24	25	1635	287	211	76	20
12	1204	212	163	49	27	26	1570	188	143	45	16
13	1075	201	152	49	15	27	1948	270	204	66	16
14	1321	379†	146	233	24	28	1658	162	126	36	10

\* 1.847 gm. of methionine were injected subcutaneously.

† 3.163 gm. of benzoylmethionine were injected subcutaneously.

‡ 1.847 gm. of methionine were given orally.

§ 1.5 gm. of cystine were injected subcutaneously.

methylation is not an uncommon reaction in the animal organism. We suggest tentatively that a primary reaction in the metabolism of methionine is demethylation to yield the next higher homologue of cysteine (homocysteine) and that 2 molecules of this compound by oxidation form homocystine which is excreted in small amounts in the urine. Confirmation of these experiments with rabbits was

obtained from studies with young white rats fed methionine by stomach tube (14). The urines of these animals gave positive cyanide-nitroprusside tests after the methionine administration.

It will be observed in Tables I and II, that normal oxidation of the sulfur of the methionine molecule in the animal organism did not occur when 1 hydrogen atom of the  $\alpha$ -amino group was replaced by a benzoyl group. The extra sulfur excreted was practically all contained in the organic sulfur fraction and there was no rise in the oxidized or sulfate sulfur fraction comparable to that observed after the administration of methionine. Further evidence that the increased excretion of organic sulfur was due to the presence of  $\alpha$ -benzoylmethionine in the urine was obtained by isolation of the compound from the urine secreted during the 6 hour period immediately following its administration. The material isolated was recrystallized and identified by its crystalline form and melting point. No depression of the melting point was observed when the recrystallized benzoylmethionine obtained from the urine was mixed with the pure substance prepared in the laboratory.

It is believed that oxidation of the sulfur of methionine, as of cystine, is related to the process of deamination of the amino acid and that if the  $\alpha$ -amino group is blocked, normal oxidation of the sulfur is impossible. No cyanide-nitroprusside reaction could be obtained from the urines excreted after the administration of  $\alpha$ -benzoylmethionine. If, as we believe, this reaction indicates the presence of homocystine in the urine as a product of the intermediary metabolism of methionine, it would appear that blocking the  $\alpha$ -amino group of methionine not only results in failure of the normal oxidation of the sulfur, but that demethylation, which we have suggested as the initial stage in the metabolism of methionine, may also be prevented.

Du Vigneaud, Dyer, and Harmon (15) have recently shown that the growth of young white rats on a cystine-deficient diet may be increased by the addition of homocystine to the diet, thus furnishing additional evidence in support of a physiological relationship between methionine and homocystine. Their results are in harmony with the suggestion that demethylation with the formation of homocystine may occur in the intermediary metabolism of methionine.



As previously stated, creatinine determinations were carried out in all the experiments. No changes beyond the limit of experimental error of the method for creatinine determination were observed. This finding is not in accord with the observations of Beard and Barnes (16), who observed an increased excretion of creatinine of 28 per cent after the feeding of 1.5 gm. of cystine to white rats. It is to be noted however that in our experiments the amount fed per kilo of body weight was much less than in the studies of Beard and Barnes. Stekol and Schmidt (9) have reported an increased excretion of creatinine after feeding 1 to 3 gm. of methionine to dogs of approximately 13 kilos in weight. It is difficult to interpret these findings. In view of the fact that many substances other than creatinine react with picric acid in the presence of sodium hydroxide to give colored complexes, it is suggested that the reaction may have been due to some product of metabolism other than creatinine.

#### SUMMARY

1. *DL*-Methionine and *L*-cystine administered in equivalent amounts orally and subcutaneously (equivalent to 400 mg. of sulfur) to rabbits were catabolized readily, the major portion of the extra sulfur appearing in the urine as oxidized (sulfate) sulfur.

2. After the administration of *DL*-methionine to rabbits, a substance having the reactions of a disulfide was excreted in the urine. This substance gave a positive cyanide-nitroprusside reaction but no test for cystine (Sullivan).

3. When the amino group of methionine was blocked as in  $\alpha$ -benzoylmethionine, oxidation by the organism of the rabbit was checked, the sulfur of the compound administered being excreted in the organic sulfur fraction of the urine. It appears that as with cystine (3-5), normal oxidation of the sulfur of methionine by the animal organism can occur only if the  $\alpha$ -amino group is free. No excretion of a compound which gave a positive cyanide-nitroprusside test was observed after the parenteral administration of  $\alpha$ -benzoylmethionine.

4. It is suggested that a primary reaction in the catabolism of methionine is demethylation and that the positive cyanide-nitroprusside tests obtained in the urine may be due to the presence of

small amounts of homocystine, formed by demethylation of methionine and oxidation of the resulting mercapto derivative to the disulfide.

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# THE CATALYTIC EFFECT OF FERRICYANIDE IN THE OXYGEN ABSORPTION OF OLEIC ACID

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Wright, Conant, and Kamerling (1) have shown that oleic acid in an alkaline potassium ferricyanide solution will absorb oxygen. They further suggested that the autoxidation of oleic acid in the presence of ferricyanide is a chain reaction.

In continuation of this problem we present in this paper studies with other catalysts with evidence for the chain reaction, and data concerning the relationship between the rate of oxygen absorption and the oxidation-reduction potentials of the catalysts.

## *Methods*

All estimations of the oxygen absorption were made with the Warburg apparatus. The composition of the phosphate buffer was 2 parts of  $m/3$   $K_2HPO_4$  and 1 part of  $m/3$   $KOH$ . Such a buffer solution has a slight advantage over 1 per cent sodium carbonate not only for its buffering capacity but also for producing a shorter induction period. Unless otherwise stated, the absorption vessel contained 2 cc. of 0.0575  $m$  oleic acid (Kahlbaum, purified grade and linoleic acid-free) solution in the phosphate buffer, 0.20 cc. of 0.115  $m$  catalyst, and 0.1 cc. of 0.023  $m$  inhibitor or of the buffer, so that the resulting solution was 0.05  $m$  in oleic acid and 0.01  $m$  in catalyst.

All experiments were performed at 25°.

## *Results*

The rate of oxygen absorption by oleic acid was catalyzed by the presence of ferricyanide, and the latter was reduced to ferrocyanide.

Oxygen was necessary for complete reduction of the ferricyanide. Thus when 100 cc. of 0.05  $m$  oleic acid and 0.01  $m$  potassium ferri-

cyanide in the phosphate buffer were stored in nitrogen freed of oxygen by bubbling through a series of Fieser (2) solutions (sodium hyposulfite plus  $\beta$ -naphthoquinone sulfonate), about 28 per cent of ferricyanide was reduced in 70 hours according to the electrometric titration of an aliquot sample with potassium molybdocyanide. Further standing in nitrogen did not increase the reduction. When oxygen was introduced over the solution, without shaking, the reduction took place with great rapidity. The results are summarized in Table I.

If more potassium ferricyanide, sufficient to restore its concentration to 0.01 M, was added to the completely reduced ferrocyanide

TABLE I  
*Rate of Reduction of Ferricyanide by Oleic Acid before and after Introduction of Oxygen*

	Time	Reduction of ferricyanide	Reduction in 1 hr.
	<i>hrs</i>	<i>per cent</i>	<i>per cent</i>
In nitrogen	20	15.4	0.77
	44	19.3	0.16
	73	28.1	0.30
	92	29.8	0.09
	116	28.4	-0.06
After introduction of oxygen	24	39.0	0.44
	48	61.0	0.92
	72	85.0	1.00
	96	94.0	0.38
	144	95.0	0.02

anide solution, more oxygen was absorbed and the added ferricyanide was reduced. On the other hand, if the solution was kept in nitrogen after the addition of ferricyanide, only a slight reduction of the latter to ferrocyanide was obtained; 9 per cent reduction after 26 hours and also 9 per cent after 74 hours. This reduction in nitrogen was probably due to some oxygen not removed. These results indicate that oxygen is necessary for the reduction of ferricyanide by oleic acid.

The ratio of oxygen utilization to ferricyanide reduction should give information on the stoichiometric relationship as well as on the length of the chain. To this end we first shook in nitrogen a 0.05 M oleic acid and 0.01 M ferricyanide solution in the phosphate

buffer, until no further reduction of ferricyanide was obtained. In two experiments the concentration of ferricyanide was reduced to 0.0076 M (Experiment A) and 0.0066 M (Experiment B). Then air was admitted into the bottle, which was connected to a gas

TABLE II  
Determination of  $O_2/K_4Fe(CN)_6$  Ratio  
Ferrocyanide produced after the introduction of oxygen.

Experiment	Time	$O_2$ absorbed	$K_4Fe(CN)_6$ produced	Ratio $O_2/K_4Fe(CN)_6$	Deviation from mean
	hrs.	mM	mM		
A	27	0.223	0.80	2.8	0.3
	50	0.558	0.22	2.5	0.0
	70	0.786	0.36	2.2	-0.3
	113	1.13	0.52	2.2	-0.3
	160	1.27	0.63	2.0	-0.5
	186	1.33	0.63	2.1	-0.4
B	24	0.29	0.22	1.3	-1.2
	48	0.65	0.22	3.0	0.45
	79	1.01	0.39	2.6	0.1
	97	1.20	0.50	2.4	-0.1
	121	1.35	0.56	2.4	-0.1
	145	1.55	0.62	2.5	0.0
	169	1.68	0.67	2.5	0.0
	193	1.87	0.67	2.8	0.3

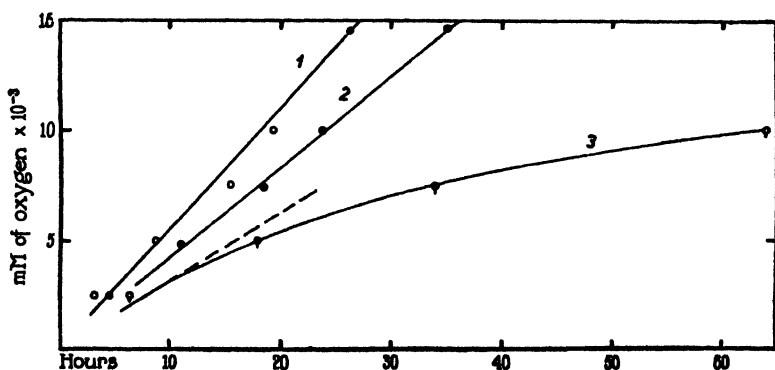


FIG. 1. The rate of oxygen absorption of 0.05 M oleic acid in phosphate buffer with different concentrations of  $K_4Fe(CN)_6$ . Curve 1, 0.10 M; Curve 2, 0.01 M; Curve 3, 0.001 M. The dotted line represents the slope of the initial part of the curve, when only a small fraction of ferricyanide is reduced.

burette and shaken. The volume of the absorbed oxygen was read, and the concentration of ferricyanide was determined by the electrometric titration. The results are given in Table II.

The average of the ratio is about 2.5. It indicates that the reduction of 2 moles of ferricyanide requires about 5 or 6 moles of oxygen.

To find how the rate of absorption is related to the concentration of the catalyst, we carried out three experiments with varying concentrations of ferricyanide. The results are plotted in Fig. 1.

The rate of absorption increases with the concentration of the catalyst. However, although the concentration of ferricyanide in Curve 1 was 100 times that in Curve 3, yet the ratio of the rates of absorption, *i.e.* the slope of the curves, is only about 3. Thus Fig. 1 clearly indicates that the rate is not proportional to the first power of concentration of ferricyanide, and the following tabulation shows that the rate is approximately proportional to the third root of the ferricyanide concentration.

Rate of O <sub>2</sub> absorption, mm O <sub>2</sub> per hr. $\times 10^3$	(K <sub>3</sub> Fe(CN) <sub>6</sub> )	(K <sub>3</sub> Fe(CN) <sub>6</sub> ) <sup>‡</sup>
	<i>M</i>	<i>M</i>
5.5	0.1	0.46
4.0	0.01	0.22
2.0	0.001	0.10

*Effect of Oxidation-Reduction Potential of Catalyst on Rate of Oxygen Consumption*—Experiments with other catalysts have shown that the oxidation-reduction potential of the catalyst is an important factor governing the rate of absorption. The potential is given by the electrochemical equation  $E = E_0 + (RT/nF) \ln ([O]/[R])$ , where  $E$  = the potential of the system,  $E_0$  = the "normal" oxidation-reduction potential characteristic of one substance, and  $[O]$  and  $[R]$  = the concentrations of the oxidant and reductant, respectively.

*Variation in Potential by Varying the Ratio, K<sub>3</sub>Fe(CN)<sub>6</sub>:K<sub>4</sub>Fe(CN)<sub>6</sub>*—It is obvious that the potential  $E$  is dependent on both  $E_0$  and the ratio of  $[O]/[R]$ .  $E_0$ , of course, is a constant for one catalyst. Oxygen absorption velocities were measured in solutions containing ferricyanide to ferrocyanide in different ratios.

The concentration of ferricyanide was constant at 0.01 M. Thus any variation in the rate must be due to the difference in potential, since potassium ferrocyanide alone does not catalyze the absorption. The results in Table III show that  $O_2$  absorption decreases with decrease in the  $Fe^{+++}:Fe^{++}$  ratio, and therefore with the oxidation potential.

*Variation in Potential by Use of a Different Catalyst*—A second method of varying the potential of the solution can be achieved by the use of a different catalyst. We have carried out experiments, using five catalysts of different normal potentials  $E_0$  (see

TABLE III

*Rate of Oxygen Absorption at Different Ratios of Potassium Ferricyanide to Ferrocyanide*

The oxygen absorbed is expressed in  $mm \times 10^3$ . The solutions were buffered with phosphate. The oleic acid concentration was 0.05 M.

$K_3Fe(CN)_6$ , mM.....	10	10	10	10	0.00
$K_4Fe(CN)_6$ , ".....	0.1	1	10	30	10
Time	Oxygen absorbed				
Hrs.					
2	3.6	2.2	0.7	0.4	
4	5.6	4.0	1.1	0.8	
6	7.2	5.4	1.8	1.3	
8	8.9	6.7	2.7	1.7	
10	10.1	8.0	3.4	2.1	0.4
15	12.0	10.7	5.3	3.1	
20	16.1	13.4	7.2	4.5	0.5
30	21.0	17.3	10.7	6.2	0.6
50	25.6	22.8	17.9	8.9	0.8

Table IV). The concentration of the catalysts, as before, is 0.01 M. Indophenol [ $O=\langle \text{benzene ring} \rangle=N\langle \text{cyclohexane ring} \rangle-O-Na$ ] having the same molarity is twice as strong as ferricyanide in the oxidizing equivalent.

The normal potentials ( $E_0$ ) are all referred to the "normal" hydrogen electrode. The  $E_0$  of the indophenol at pH 11 of the phosphate buffer is  $-0.08$  volt. The normal potentials of the copper-glycine complex and of the copper-pyridine complex at pH 11 were measured electrometrically by the method of titration with sodium hydrosulfite in the phosphate buffer. The copper complexes were



prepared by adding 0.01 mM of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  to the phosphate buffer, 0.05 M in oleic acid and 0.05 M in pyridine or glycine respectively. In the determination of  $E_0$ , oleic acid was omitted. The average value of the normal potentials of the two complexes is 0.01 volt.

Table IV is arranged in such a way that the normal potential decreases from left to right. The rate also decreases in the same direction.

Since the copper complexes and the indophenol have very low potentials, the atmospheric oxygen will oxidize their reduced forms

TABLE IV  
*Relationship between Rate of Absorption and  $E_0$  of Different Catalysts*  
The oxygen absorbed is expressed in  $\text{mm} \times 10^3$ .

Time	Control, no catalyst	$\text{K}_2\text{Mo}(\text{CN})_6$ $E_0 = +0.72$ volt	$\text{K}_3\text{Fe}(\text{CN})_6$ $E_0 = +0.44$ volt	Cu-glycine $E_0 = +0.01$ volt	Cu-pyridine $E_0 = +0.01$ volt	Indophenol $E_0 = -0.08$ volt at pH 11
<i>hrs.</i>						
$\frac{1}{4}$		1.9				
$\frac{1}{2}$		3.3				
1		6.8	1.8	0.4	0.4	0.2
2		12.2	3.6	0.9	0.8	0.4
4	0.1	13.4	5.7	1.8	1.4	1.1
6			7.6	2.7	2.4	1.8
8			9.3	3.6	3.0	2.6
10	0.2		10.7	4.4	3.6	3.1
15			13.7	6.4	5.3	4.8
20	0.2		16.0	8.9	7.2	6.6
30			21.0	13.0	11.0	10.2
50	0.4		25.0	22.3	17.9	16.1

as soon as they are formed. For that reason, the concentration of catalysts in the oxidized form is practically constant during the oxygen absorption by oleic acid. If this is true, the rate of absorption will be constant, as long as oxygen and oleic acid are in excess. Thus we would expect to get a straight line, if we plot the oxygen absorbed against time. This is found essentially to be the case in Fig. 2. For comparison, the results with ferricyanide as catalyst are also included. The ferricyanide curve shows definite decrease in rate as the concentration of ferricyanide diminishes. The copper complexes and the indophenol curves give essentially

constant slopes. Control experiments on pyridine and glycine (*i.e.* without  $\text{CuSO}_4$ ) are included in Fig. 2 to show the increase of the catalytic effect by the presence of copper sulfate. The formation of the copper complexes is indicated by the characteristic deep blue color.

Potassium molybdicyanide is metastable to the oxygen electrode at pH 11. The decomposition is catalyzed by light; therefore the molybdicyanide experiment (Table IV) was performed in

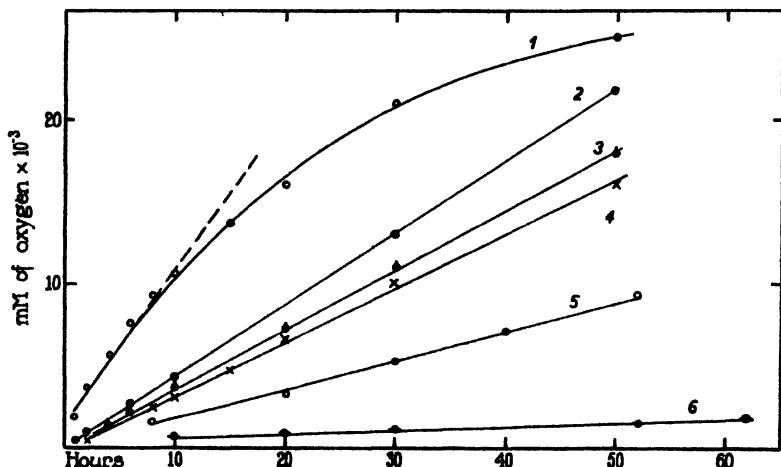


FIG. 2. The rate of oxygen absorption of 0.05 M oleic acid in phosphate buffer under the influence of 0.01 M catalysts. Curve 1, ferricyanide; Curve 2, copper-glycine complex; Curve 3, copper-pyridine; Curve 4, indophenol; Curve 5, 0.05 M pyridine; Curve 6, 0.05 M glycine. The dotted line represents the slope of the initial part of the curve, when only a small fraction of ferricyanide is reduced.

the dark. Even then decomposition took place. For that reason no measurements after 4 hours were recorded.

**Reproducibility of Results**—During the whole course of the work one sample of pure oleic acid was used. Other reagents were prepared from time to time. In some thirty experiments with potassium ferricyanide the results checked with each other within 10 per cent or less. Data in Tables III and IV are the typical results. In three experiments with indophenol the results also checked with one another within less than 10 per cent.

The results of copper complexes are more variable. Their catalytic activities are much more affected by impurities. Impure reagents gave higher rates. To avoid as much as possible the presence of impurities, the phosphate buffer was prepared from Kahlbaum's highest grade potassium hydroxide and phosphoric acid. Copper sulfate was repeatedly crystallized. The ordinary distilled water was fractionated several times. Pyridine was distilled over pure potassium hydroxide and refractionated. The results of the oxygen absorption obtained by using ordinary and purified reagents are presented in Table V.

TABLE V

*Difference between Rates of Oxygen Absorption Catalysed by Ordinary and by Carefully Purified Copper-Pyridine Complexes*

The figures represent oxygen absorbed in  $\text{mm} \times 10^3$ .

Time, hrs.....	5	10	20	40
Ordinary reagents	3.1	6.2	12.5	25.0
	3.1	6.2	10.7	22.8
	3.0	5.6	11.6	17.9
Purified reagents	2.2	3.6	7.2	14.3
	2.1	3.5	6.7	12.5
	1.8	4.0	6.9	12.5
	1.8	3.4	6.3	12.5
	1.9	4.0	6.9	12.5
	1.8	3.8	6.7	13.0
	1.9	3.8	6.9	13.2

The experiments with the purified reagents were performed at seven different times. The results, as given in Table V, show a reasonable accuracy and reproducibility. Whether the higher rate with the ordinary reagent is due to impurities or not is an open question.<sup>1</sup> Nevertheless, the experiments with the purified reagents apparently show the catalytic effect of the copper-pyridine complex.

<sup>1</sup> The fact that purified pyridine is also a catalyst may also be due to impurities. The presence of  $0.000002 \text{ M}$  KCN completely inhibits the oxygen absorption.

*Inhibitors*

Preliminary work (1) indicated that the reaction rate was decreased by the addition of compounds known to function as inhibitors of chain reactions. Further experiments of this kind are now reported. Obviously the inhibitors for the oxygen absorption by oleic acid in the presence of potassium ferricyanide must not reduce the catalyst. For that reason, the inhibitors such as hydroquinone, pyrogallol, etc., which are extremely effective in other chain reactions cannot be used. We have tried several inhibitors on the absorption catalyzed by ferricyanide and copper-pyridine complex. Because of the limitation stated above, the

TABLE VI

*Results of Inhibition on Ferricyanide Catalyst*

The figures represent oxygen absorbed in  $\text{mm} \times 10^3$ .

Time (1)	Phenol 0.001 M (2)	Saturated solution of dimethyl- aniline 0.001 M (ca.) (3)	Ethanol amine 0.001 M (4)	Eugenol 0.001 M (5)	0.005 M $\text{K}_3\text{Fe}(\text{CN})_6$ 0.005 M $\text{K}_2\text{Fe}(\text{CN})_6$ 0.050 M oleic acid (6)	None (7)
hrs.						
5	2.7	2.4	2.7	3.1	2.7	6.7
10	4.9	4.5	4.5	4.5	4.5	10.7
15	7.1	5.8	6.2	6.9	5.8	13.7
20	8.9	7.3	7.4	7.5	6.9	16.0
30	11.7	9.4	9.0	10.7	7.8	21.0
50	14.3	11.2		12.5	11.6	25.0
70	17.0	15.3		14.8	12.0	

inhibition effects obtained are not as pronounced as in reactions definitely known to have a chain mechanism.

A control experiment was performed to follow the reduction of ferricyanide by the inhibitors (0.001 M) alone without the presence of oleic acid. In 20 hours not more than 25 per cent of ferricyanide was reduced, as determined by electrometric titration with molybdicyanide. Another experiment was carried out with 0.05 M oleic acid in the buffer solution which contained both  $\text{K}_4\text{Fe}(\text{CN})_6$  and  $\text{K}_3\text{Fe}(\text{CN})_6$  in 0.005 M concentration. These would be the concentrations if an inhibitor had reduced 0.01 M ferricyanide 50 per cent at the very beginning of the experiment. Column 6 of Table

VI gives the result of absorption of such a solution. The comparison between this column and the inhibitor columns (Nos. 2 to 5) gives evidences that inhibition is real and not due to the decrease of ferricyanide concentration. Two points are worth mentioning in this connection: (1) If there were no real inhibition, the rates shown by the inhibitors during the first 10 hours could be accounted for by the reduction of ferricyanide only of 50 per cent or more. Such reduction did not occur, since the electrometric titration of the control sample with potassium molybdicyanide showed that only about 10 per cent ferrocyanide was produced in 10 hours. (2) In Column 6 after 50 hours the absorption was very slight

TABLE VII  
*Inhibitors of Copper-Pyridine Catalyst*

The oxygen absorbed is expressed in  $\text{mm} \times 10^3$ .

Time <i>hrs</i>	None	Dimethylaniline		<i>p</i> -Bromo phenol 0.001 M	Phenol	
		Solution A*	Solution B†		0.001 M	0.0001 M
5	2.2	0.4	0.9	0.8	0.8	1.7
10	3.6	1.3	1.1	1.3	1.8	3.3
20	7.2	1.7	2.2	2.7	3.8	5.8
40	14.3	2.0	4.2	4.5	8.3	11.6
70	24.8	2.9	5.8	7.2	14.3	20.6
100		3.6			17.8	

\* Solution A = saturated solution of dimethylaniline in the phosphate buffer.

† Solution B = Solution A diluted 10-fold with buffer.

and an end-point of  $12.0 \times 10^{-3}$  mm of oxygen was soon reached. The electrometric titration of the solution after 70 hours gave the presence of ferricyanide in less than 5 per cent. On the other hand, the rate of absorption of inhibitor solutions was not changed considerably. The yellow color of ferricyanide in the solutions still existed.

Since the copper-pyridine complex has a very low oxidation-reduction potential, there is no possibility of complication from reduction of  $\text{Cu}^{++}$  by the inhibitors. Hence the evidence of inhibition is especially definite in the case of this catalyst. The results are given in Table VII.

Dimethylaniline apparently has a greater inhibiting power than either one of the phenols.

Besides oleic acid other unsaturated compounds have been tried. Preliminary experiments have shown that linoleic acid and linseed oil suspended in the phosphate buffer absorbed oxygen much more rapidly than oleic acid when equal concentration of ferricyanide was present. However, undecylenic acid under the same conditions absorbed oxygen more slowly than oleic acid.

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## THE OXIDATION OF THE STEREOISOMERS OF CYSTINE IN THE ANIMAL BODY

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It has recently been shown that *d*-cystine is unable to support growth in lieu of the naturally occurring *l*-cystine (1). This is quite in contrast to the behavior shown by the isomers of either tryptophane or methionine. The dextro isomer of tryptophane can replace the naturally occurring levo isomer (2, 3) while the same relationship holds true for *d*- and *l*-methionine (4).

In order to explain the utilization of *d*-tryptophane we suggested (3) that the compound is oxidatively deaminized in the body to the keto acid in accordance with the generally accepted view of the catabolism of amino acids and that the keto acid is then asymmetrically converted into *l*-tryptophane. That such a reaction is possible is strongly indicated by the work of Knoop and coworkers (5-7), who furthermore adduced evidence that the conversion of keto acid to amino acid proceeds through the acetyl derivative of the amino acid. We have recently obtained confirmatory evidence of this hypothesis of Knoop's. The findings of Jackson (8) and of Berg, Rose, and Marvel (9) that indolepyruvic acid can replace *l*-tryptophane indicate that the body can convert the keto acid to the amino acid and are in harmony with the above explanation of the replaceability of *l*-tryptophane by the dextro isomer. The lack of availability of *d*-cystine takes on, therefore, added significance in relation to the intermediary metabolism of cystine.

Since oxidative deamination seems to occur regardless of spatial configuration, as indicated by the work with the other amino acids studied, one might expect that this would hold true as well for cystine if oxidative deamination is in reality the primary step in



its catabolism. The fact that *d*-cystine does not replace *l*-cystine might be due simply to the instability of the keto acid and therefore opportunity for conversion to the amino acid is not afforded. But if this were the case, *d*-cystine should be as readily oxidized as *l*-cystine, since the keto acid formed from both enantiomorphs would be identical. On the other hand, if the oxidation of *d*-cystine should be found to be more difficult or less complete, then it seems reasonable that the primary oxidative step in the catabolism of cystine may not be exclusively an oxidative deamination. A study of the comparative oxidation of *d*- and *l*-cystine in the animal body was therefore indicated.

A study of the oxidation of *dl*-cystine and mesocystine was also desirable from certain standpoints which will be discussed later in this paper.

#### EXPERIMENTAL

Rabbits weighing from 2.5 to 3 kilos were employed as the experimental animals. They were kept in metabolism cages on a definite daily food intake which was determined from a fore period during which they ate *ad libitum*. The amount of food given was then limited so that the rabbits would clean up their ration each day. The diet used was Purina Rabbit Chow. Analyses were carried out on 24 hour specimens of urine. At a definite time each morning the bladder was emptied by gentle pressure upon the abdomen and the urine thus obtained added to any urine that may have been collected from the cage. Admixture of feces with urine in the cage was avoided by means of a double false bottom. The first screen was sufficiently coarse to allow the ready passage of feces to the second screen which was fine enough to prevent their dropping into the collecting pan.

Total sulfur was determined by Denis' (10) modification of Benedict's procedure (11). Total and inorganic sulfates were estimated by the Folin (12) methods. Total nitrogen determinations were carried out by a micro-Kjeldahl method (13). The cystine administered was given in water suspensions by means of a stomach tube before the daily ration was placed in the cage.

The individual experiments with the stereoisomers are given in Tables I to III. In the experiments recorded in Table I on Rabbits A and B, the total sulfur and inorganic sulfate sulfur were

TABLE I  
*Oxidation of d- and l-Cystine*

	Day	Total N	Total S	Total SO <sub>2</sub> -S	Inorganic SO <sub>2</sub> -S	Ethereal SO <sub>2</sub> -S	Unoxidised S	Cystine fed
		gm.	mg.	mg.	mg.	mg.	mg.	gm.
Rabbit A, 2.4 kilos; daily diet 105 gm. Rabbit Chow	1	1.60	160	130	106	24	30	0.5 l-
	2	1.60	154	121	118	3	33	
	3	2.00	146	128	105	23	18	
	4	1.80	254	210	190	20	44	
	5	1.80	144	114	107	7	30	
	6	1.80	151	129	117	12	22	
	7	1.87	146	116	109	7	30	
	8*	2.00	163	124	109	15	39	
	9*	2.00	163	124	109	15	39	
	10	1.95	258	172	155	17	86	
Rabbit B, 2.6 kilos; daily diet 110 gm. Rabbit Chow	11	1.60	138	106	99	7	32	0.5 d-
	1	1.60	150	111	91	20	39	
	2	1.40	150	130	114	16	20	
	3		147	116	99	17	31	
	4	1.70	244	169	145	24	75	
	5	1.70	141	100	95	5	41	
	6	1.80	147	125	110	15	22	
	7	1.80	139	102	97	5	37	
	8*	2.00	149	119	103	16	30	
	9*	2.00	149	119	103	16	30	
	10	1.70	254	157	150	7	97	
	11	1.60	144	110	98	12	34	

\* Based on a 48 hour sample.

TABLE II  
*Oxidation of Cystine Isomers*

Day	Total N	Total S	Total SO <sub>2</sub> -S	Unoxidised S	Cys-tine fed	Day	Total N	Total S	Total SO <sub>2</sub> -S	Unoxidised S	Cys-tine fed
	gm.	mg.	mg.	mg.	gm.		gm.	mg.	mg.	mg.	gm.
1	1.70	136	109	27	0.5 d-	54	2.10	157	132	25	0.5 Meso-
2	1.90	149	119	30		55	1.94	159	122	37	
3	1.90	149	119	30		56	1.90	165	132	33	
4	1.90	138	118	20		57	2.20	265	207	58	
5	1.83	229	149	80		58	2.00	171	125	46	
6	1.75	141	108	33		59	2.10	164	133	31	
10	1.85	151	123	28		88	1.70	146	123	23	
11	1.75	151	120	31		89	1.70	142	119	23	
12	1.71	155	120	35		90	1.75	148	119	29	
13	1.84	279	226	53	0.5 l-	91	1.92	153	129	24	0.5 dl-
14	1.68	141	116	25		92	1.90	250	189	61	
15	1.75	144	120	24							

determined in order to find out if the ethereal sulfates varied after feeding the cystine. Since no significant changes were observed, they were not determined in the later experiments. The nitrogen

TABLE III  
*Oxidation of Cystine Isomers*

Day	Cystine fed	Total S	Total SO <sub>4</sub> -S	Unoxidised S	Day	Cystine fed	Total S	Total SO <sub>4</sub> -S	Unoxidised S
Rabbit D, 2.2 kilos; daily diet 100 gm. Rabbit Chow					Rabbit E, 2.5 kilos; daily diet 90 gm. Rabbit Chow				
	gm.	mg.	mg.	mg.		gm.	mg.	mg.	mg.
1		127	104	23	1		118	96	22
2		134	111	23	2		123	103	20
3*		144	119	25	3		128	98	30
4*		144	119	25	4		111	89	22
5	0.5 d-	214	148	66	5		98	79	19
6		111	94	17	6	0.5 l-	232	180	52
7		117	96	21	7		96	79	17
12		121	86	35	8		107	79	28
13		101	88	13	Rabbit F, 2.3 kilos; daily diet 100 gm. Rabbit Chow				
14		127	94	33	1		149	112	37
15	0.5 l-	224	188	36	2		151	111	40
16		108	86	22	3		140	114	26
17		113	84	29	4	0.5 Meso-	242	182	60
20		129	101	28	5		144	110	34
21		103	93	10	6*		144	119	25
22		93	78	15	7*		144	119	25
23	0.5 Meso-	209	154	55	8		163	117	46
24		100	88	12	9		134	114	20
Rabbit B, 2.7 kilos; daily diet 110 gm. Rabbit Chow					10	0.5 dl-	240	183	57
1		144	110	34	11		152	119	33
2		153	118	35					
3	0.5 l-	241	187	54					
5*		147	117	30					
6*		147	117	30					
7	0.5 Meso-	242	170	72					

\* Based on a 48 hour sample.

was determined daily in all cases in order to detect any exaggerated protein catabolism which might have resulted from the feeding of some of these isomers. The nitrogen values are given in

Tables I and II to show that this did not occur and for the sake of brevity are omitted in Table III.

TABLE IV  
*Percentage Oxidation of Cystine Isomers*

Rab- bit	Cystine adminis- tered	Total S	Average total S of fore period	In- crease in total S	SO <sub>2</sub> -S found	Average SO <sub>2</sub> -S of fore period	In- crease in SO <sub>2</sub> -S	In- creased S as SO <sub>2</sub>	Admin- istered S ox- idised	S re- covered
		mg.	mg.	mg.	mg.	mg.	mg.	per cent	per cent	per cent
A	<i>l</i> -	254	153	101	210	126	84	83	63	76
B	"	241	149	92	187	114	73	79	55	69
C	"	279	152	127	226	121	105	83	79	95
D	"	224	116	108	188	89	99	92	74	81
E	"	232	116	116	180	93	87	75	65	87
Average.....				109			89	82	67	82
A	<i>d</i> -	258	153	105	172	121	51	49	38	79
B	"	244	149	95	169	119	50	53	38	71
B	"	254	145	109	157	113	44	40	33	82
C	"	229	143	86	149	116	33	38	25	65
D	"	214	137	77	148	113	35	45	26	58
Average.....				94			42	45	32	71
B	Meso-	242	147	95	170	117	53	56	40	71
C	"	265	160	105	207	129	78	74	58	79
D	"	209	108	101	154	91	63	62	47	76
F	"	242	147	95	182	112	70	74	53	71
Average.....				99			66	67	50	74
C	<i>dl</i> -	250	147	103	189	122	67	65	50	77
F	"	240	146	94	183	116	67	71	50	71
Average.....				99			67	68	50	74
Average of experiments with <i>d</i> - and with <i>l</i> -cystine.....							66	64	50	77

The data from the individual experiments are summarized in Table IV together with the calculations of the percentage oxidation of the compounds. The increase in total sulfur was obtained from the difference between the average value of the total sulfur

during the fore period and the values found after feeding the compound. The increase in total sulfates was obtained in a similar way. The percentage of oxidation is expressed in terms of the increase in total sulfur excreted as well as in terms of the sulfur administered. It was expressed first as the percentage of extra sulfur which appears as sulfate and secondly, by the percentage of the total sulfur administered which appears as extra total sulfate.

In order to see if any of the large amount of unoxidized sulfur obtained after feeding of *d*-cystine represented unchanged *d*-cystine, the urine from one of the animals (Rabbit C) was analyzed for cystine by Sullivan's method (14) both before and after hydrolysis with HCl. An amount of cystine was found which corresponded to about one-third of the extra unoxidized sulfur. The same value within experimental error was found both before and after hydrolysis.

#### DISCUSSION

The data presented in Tables I to III show very definitely that the dextro isomer is not as readily oxidized as the naturally occurring *l*-cystine. After the feeding of *l*-cystine about 82 per cent of the extra sulfur in the urine was sulfate sulfur, while after *d*-cystine only about 45 per cent of the extra sulfur appeared in this form. The racemic isomer, as one might expect, was oxidized at an efficiency about midway between that of the two active isomers as brought out in Table IV.

The mesocystine was oxidized to the same extent as the racemic isomer. This is clearly shown in Table IV. A reasonable explanation of this finding would be that the mesocystine had been reduced to *d*- and *l*-cysteine and that these had then been oxidized to the same extent as *d*- and *l*-cystine respectively. The likelihood of this preliminary reduction of mesocystine was also pointed out in our growth studies with this compound (15). That such a reduction occurs in the catabolism of *l*-cystine is furthermore strongly indicated by the researches of Lewis and coworkers (16 to 18) and by Sherwin, Shipley, and Rose (19).

It is difficult to explain the limited oxidation of *d*-cystine on the assumption that the first step in the oxidation of cystine is exclusively an oxidative deamination. As pointed out earlier it appears that oxidative deamination is not generally dependent on

stereostructure, yet if this were the case one would expect to find most of the neutral sulfur in the urine in the form of cystine or cysteine, which is actually not the case. It is equally difficult to explain the oxidation of cystine entirely on the basis of a preliminary oxidation of the sulfur end of the molecule, for the investigations of Lewis and coworkers (16, 18) and of Sherwin, Shipley, and Rose (19) point very strongly to the importance of a potentially free amino group in cystine for the complete oxidation of the sulfur. It is of particular interest in this connection that after the injection of benzoylcystine Lewis, Updegraff, and McGinty (18) were able to account for practically all of the sulfur as sulfhydryl or disulfide, indicating that no oxidation had occurred.

With cystine playing a part, however, in so many reactions of the body and from a structural standpoint in different ways, it would not be surprising if its breakdown did not proceed exclusively by one route. A part of the cystine might be subjected to oxidation by way of oxidative deamination of the amino group while another fraction might be catabolized by way of oxidation of the sulfur end of the molecule. If this were the case we should expect that the cystine oxidation proceeding by oxidative deamination would not be dependent on stereostructure and would proceed to the keto acid, and that the latter, being extremely unstable, would quickly decompose so that resynthesis to *L*-cystine and utilization for growth could not take place. On the other hand the oxidation involving the sulfur as the primary point of attack might be of an irreversible type and might yield a product whose further breakdown was dependent upon its stereostructure. This product would then be excreted in the urine in the partially oxidized state and would be determined as neutral sulfur. This would explain the partial oxidation of *D*-cystine and also its failure to support growth on a cystine-deficient diet. The partial oxidation of benzylcysteine found by Sherwin, Shipley, and Rose (19) might be explained along similar lines.

That the difference between the oxidation of the isomers is not simply a question of speed of oxidation is indicated by a comparison of the results on *D*-cystine and *DL*-cystine. If it were simply a question of the rate of oxidation, one might expect that the feeding of smaller amounts of *D*-cystine would lead to a more complete oxidation. This, though, seems not to be the case. In the experi-

ments on *dl*-cystine and mesocystine the amount of potential *d*-cystine was only half that given in the experiments on *d*-cystine, yet the *d*-cystine was evidently no better oxidized at this lower level than at the higher level. The same percentages of the *d*-cystine appeared as neutral sulfur.

The results on *dl*-cystine and mesocystine are apparently not in agreement with the conclusions of Hele and Pirie (20) and of Lawrie (21) from their experiments on dogs and rats respectively. These investigators came to the conclusion that the dog and rat could oxidize *dl*-cystine as readily as the *l*-cystine. Our experiments were carried out with rabbits and although it is rather unlikely, there may be a species difference. Although the inactive cystine used by these workers was without doubt a mixture of *dl*-cystine and mesocystine, this should not in itself have affected the results, since we have obtained the same degree of oxidation with both inactive isomers. Furthermore in some of their experiments inactive cysteine was employed and of course in that case the possibility of the presence of a meso form is excluded. There seems to be however some indication, although slight, that the *dl*-cysteine is not as well oxidized as the *l*-cysteine. In their experiments the average  $\text{SO}_4\text{-S}$  is less after *dl*-cysteine than after *l*-cysteine. The average  $\text{SO}_4\text{-S}$  percentage from all four experiments is 62.5 per cent for *dl*-cysteine and for three experiments on *l*-cysteine it is 67.5 per cent. Of possibly greater significance is the fact that considerably more neutral sulfur was excreted after the feeding of *dl*-cysteine, as was pointed out by the authors themselves. The appearance of large amounts of neutral sulfur in the urine after the feeding of inactive cystine was also the case in Lawrie's investigations.

It might be advisable to point out here that in Hele and Pirie's paper the reduced *l*-cystine is spoken of as *d*-cysteine, presumably because of the dextrorotation of the cysteine hydrochloride. We should like to suggest that there may be some chance for confusion in so doing and that it might be preferable to call the reduced form of *l*-cystine, *l*-cysteine. This seems to be all the more justifiable since the compound itself, in aqueous solution, has a levorotation (22).

#### SUMMARY

The oxidation of the stereoisomers of cystine has been studied. It has been shown that *d*-cystine is much more difficultly oxi-

dized than *l*-cystine. Mesocystine and *dl*-cystine have been shown to be oxidized to a degree midway between that of *l*- and *d*-cystine.

The possible significance of these findings to the theories on the intermediary metabolism of cystine has been discussed.

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## NECESSARY VERSUS OPTIMAL INTAKE OF VITAMIN G (B<sub>2</sub>)

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In accordance with the general ambition of modern science to make itself more and more exact, investigation of the vitamins, while developing qualitatively toward completion of the list of nutritionally essential substances and the chemical identification of each, is also being developed quantitatively. Such quantitative research seeks (among other things) to ascertain what amounts or proportions yield the best results in nutrition, and to what extent a nutritional status which meets our present concept of the normal may still be improved by quantitative adjustments of the chemical factors of an already adequate food supply.

Mellanby and Green, in their well known work with vitamin A values, found that the intake required to insure optimal vitality as indicated by the criteria developed in their research was about 4-fold the intake which sufficed for normal growth with complete protection from any outwardly visible sign of specific deficiency; and Batchelder (data in preparation for publication) finds that in carefully quantitative experiments covering most or all of the life cycle, the measured results in normal nutrition continue to show improvement (either in the original test animals or their offspring) with increasing intakes of vitamin A up to levels of intake much above what is qualitatively demonstrable as strictly necessary.

An apparently analogous situation exists in the case of vitamin C, in which clinical observations and laboratory experiments have indicated that nutritional well being continues to improve with increasing intake of vitamin C up to levels well above what is necessary for the prevention of manifest scurvy.

*Nature of Present Work*—In the experiments briefly described in the present paper, vitamin G ( $B_2$ ) was fed at four different levels of intake, while all other known nutritional factors were maintained at a constant level in the food mixture. Only at the lowest of these levels of vitamin G intake was there visible indication of nutritional inferiority, and even here one could not say that there was any characteristic sign of vitamin G deficiency. Only between this first (lowest) and the second level of intake was there a pronounced difference in the average weight curve or any difference in the physical appearance of the animals. Yet further increases in the intake of vitamin G, while without qualitatively apparent influence, were found to result in improved nutritional well being when the studies were conducted by sufficiently comprehensive and quantitative methods.

#### EXPERIMENTAL

The experiments here recorded were conducted in two series. Series I consisted of a direct comparison of three diets fed to rats 28 days old at the beginning of the experiment; the members of each litter were distributed as evenly as possible to each diet, and the distribution of successive litters so planned that the total populations assigned to the different diets were evenly matched as to sexes and initial size. These three diets, alike in their contents as to all other known nutritional essentials, contained respectively 0.9, 1.3, and 2.2 Bourquin-Sherman units (1) of vitamin G per gm. of air-dry food mixture. The animals were kept in metal cages floored with wire screens, so that they had no opportunity to gnaw wood or eat bedding. Series II was started at a later date, when the early results of the first series had made apparent the desirability of another comparison at a lower level of vitamin G intake. The selection and matching of experimental animals was as described for Series I, but as a further precaution (lest coprophagy should become a factor when the vitamin G intake was so low) the cages of the animals of Series II were floored with screens of larger ( $\frac{3}{4}$  inch) mesh, raised 1 inch above the trays which received the feces. The diets fed in Series II were like those of Series I in their concentrations of other nutrients and contained 0.4 unit and 0.9 unit of vitamin G per gm. of air-dry food, respectively. The overlapping of the diets of the two series

at the level of 0.9 unit per gm. served to ensure that the experiments at the four levels of vitamin G intake were comparable; *i.e.*, that the special precautions against coprophagy were not necessary when the food furnished enough vitamin G to provide amply for the needs of normal growth.

Vitamin G was furnished in the form of skim milk powder, the respective proportions of which in the food mixtures were such as to yield the gradations of vitamin G values just stated. The proportions of the other ingredients of the food mixtures were then adjusted with the view to supplying the same concentrations of proteins, fats, carbohydrates, mineral elements, and vitamins

TABLE I  
*Percentage Composition of Diets in Terms of Ingredients Used*

	Diet 1 0.4 unit of vitamin G per gm. (Laboratory No. 116)	Diet 2 0.9 unit of vitamin G per gm. (Laboratory No. 92)	Diet 3 1.3 units of vitamin G per gm. (Laboratory No. 106)	Diet 4 2.2 units of vitamin G per gm. (Laboratory No. 206)
Skim milk powder.....	2.5	7.5	15.0	30.0
Casein (purified).....	14.5	12.8	10.2	5.1
Osborne-Mendel* salt mixture.....	3.7	3.4	2.9	1.8
Butter fat.....	9.1	9.1	9.1	9.0
Cod liver oil.....	1.0	1.0	1.0	1.0
Corn-starch with adjusted amount of vitamin B.....	69.2	66.2	61.8	53.1

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

A, B, and D in each of the four diets. The potency of the alcoholic extract of whole wheat which was used in adjusting the vitamin B values of the diets was measured by the method of Chase and Sherman (2). The calculated amount of the extract was then administered by evaporating upon corn-starch and incorporating this "activated" starch into the diet. For the convenience of the reader the four diets are here designated Diets 1, 2, 3, and 4 in order of *increasing* vitamin G value. The general composition of these diets is shown in Table I.

Vitamin G was here furnished in natural food form both because in the state of knowledge existing when these experiments were begun the attempt to devise a more synthetic diet for use over

so large a part of the life cycle might have led to deficiencies of unknown essential substances and because sufficient amounts of purified concentrates to feed the necessary numbers of animals for such lengths of time would have been prohibitively expensive to prepare. In a further development of this field of research we hope to vary the intake of vitamin G as a more nearly isolated factor, through a wider range, and to continue the work throughout the life cycle of the experimental animals.

Observations covered by the experiments here reported included (1) the gains in weight during the 28th to 56th days of life, both by the original animals assigned to these diets at 28 days of age and by an equal or larger number of their offspring; (2) the breeding record, including ages of the females at the birth of their first

TABLE II

*Growth from 28th to 56th Day of Life, with Increasing Intakes of Vitamin G*

Diet No.	Vitamin G value per gm.	Males			Females		
		No. of rats	Gain ( $\pm$ probable error)	Difference ( $\pm$ probable error)	No. of rats	Gain ( $\pm$ probable error)	Difference ( $\pm$ probable error)
	units		gm.	gm.		gm.	gm.
1	0.4	22	38.6 $\pm$ 1.6		29	31.5 $\pm$ 1.0	
2	0.9	23	65.4 $\pm$ 1.9	26.8 $\pm$ 2.4	34	53.3 $\pm$ 0.6	21.8 $\pm$ 1.1
3	1.3	21	80.1 $\pm$ 1.8	14.7 $\pm$ 2.6	23	64.4 $\pm$ 0.9	11.1 $\pm$ 1.1
4	2.2	22	95.5 $\pm$ 1.3	15.4 $\pm$ 2.2	24	71.4 $\pm$ 1.1	7.0 $\pm$ 1.4

litters (the sexes having been caged together so that breeding would take place as the requisite degree of maturity was reached), duration of reproductive life, and success in the bearing and rearing of young; (3) average weights of young at a standard weaning age of 28 days; (4) abilities of representative 28 day-old rats from the different diets to gain weight on vitamin G-free diets by virtue of their bodily stores of vitamin G.

#### DISCUSSION

1. On the diet which furnished 0.4 unit of vitamin G per gm. of air-dry food, growth was below the average shown by rats of our colony on thoroughly satisfactory diets; on the diet with 0.9 unit, the growth approximated this average; enrichment to 1.3

units of vitamin G value per gm. showed a slightly higher rate of gain; and with further enrichment to 2.2 units, growth was again slightly increased. From Table II it may be seen that these results are true of both sexes and that the differences due to even the last increase of the vitamin G value of the diet are 5 to 7 times their probable errors and are therefore undoubtedly significant.

Thus increases of intake of vitamin G above the level which fully meets the requirements of actual need are shown to result in successive increases in the rate of growth.

2. The successive enrichments of the diet in its vitamin G value, which thus induced moderate increases in the rate of growth, resulted also in an improved rate of development. This is illustrated in the fact that the females on Diet 3 bore young at an

TABLE III

*Influence of Level of Vitamin G Feeding upon Weights of Young at Late Infancy (Young Rats at 28 Days of Age)*

Diet No.	Vitamin G value per gm.	Males			Females		
		No. of rats	Gain ( $\pm$ probable error)	Difference ( $\pm$ probable error)	No. of rats	Gain ( $\pm$ probable error)	Difference ( $\pm$ probable error)
	units		gm.	gm.		gm.	gm.
1	0.4	20	33.9 $\pm$ 0.7		32	33.6 $\pm$ 0.7	
2	0.9	140	38.8 $\pm$ 0.3	4.9 $\pm$ 0.8	143	38.7 $\pm$ 0.3	5.1 $\pm$ 0.7
3	1.3	123	47.4 $\pm$ 0.5	8.6 $\pm$ 0.6	115	44.4 $\pm$ 0.4	5.7 $\pm$ 0.5
4	2.2	89	51.0 $\pm$ 0.6	3.6 $\pm$ 0.7	109	48.8 $\pm$ 0.5	4.4 $\pm$ 0.7

average of 22 days earlier than those on Diet 2. That this was a true improvement in rate of development and not an acceleration forced at the expense of vitality is shown by the fact that the individuals receiving the higher intake of vitamin G and showing increased rates of growth and development showed also increased vitality in their adult lives as evidenced by an extension of the period between the attainment of maturity and the onset of senility, by greater success in the production and rearing of young, and by the further fact that the young of these animals were superior to the cousins born of parallel animals whose food was less rich in vitamin G. As between Diets 1 and 2 the differences resulting in these respects were unmistakable. The further improvements resulting from further increases of vitamin G

intake are naturally less conspicuous and for the populations of the size which we could include in the experiments here reported (12 to 14 females each on Diets 3 and 4) the data are not yet statistically conclusive; but it is logically most probable that the observed differences are real and are due to the one known variable factor; *i.e.*, to the successive increments of the vitamin G intake.

3 With the much more numerous data on weights of young at 28 days of age, it is plain that the results are statistically significant. As summarized in Table III they show that a decided improvement results from the feeding of Diet 3 as compared with Diet 2. On Diet 4 as compared with Diet 3 there is a further gain of from 5 to 7 times its probable error, which is undoubtedly significant when viewed in the light of the findings at the other levels.

4. The ability of the young of a given age (in this investigation, 28 day-old rats) to grow for a time on a diet otherwise good but lacking vitamin G was found to be markedly influenced by the level of vitamin G value in the dietaries of the families to which they belonged. Those from Diet 1 (0.4 unit of vitamin G value per gm. of air-dry food) lost weight; those from Diet 2 (0.9 unit) showed an average gain of 3.8 gm. in 6 weeks; those from Diet 3 (1.3 units), 6.4 gm.; those from Diet 4 (2.2 units), 10.3 gm. of gain in the same 6 week period. While the numbers of cases, 6 to 14 at each level, are hardly large enough to warrant statistical treatment, the differences are so clear cut and consistent as to make it highly probable that still further gains may be realized at still higher levels of vitamin G intake.

#### SUMMARY

Food mixtures equally rich in other known nutrients and with vitamin G values adjusted to four different levels were fed to experimental animals (rats) from the end of infancy to the completion of the breeding record and the effects of the differing vitamin G intakes upon nutritional well being and vitality were observed throughout this part of the life cycle, and also upon the offspring.

The improvement which here resulted from the enrichment in vitamin G of an already adequate diet was chiefly apparent in the superior vitality of the young and the extension of the

period between the attainment of maturity and the onset of senility.

There was increased growth both of the original young animals and of their offspring, though all of the growth rates involved in these experiments were well below the records of very rapid growth which have been observed by other investigators in recent years.

In the experiments here recorded, the moderate increases in growth rate with increasing intake of vitamin G were evidently related to increases of vitality. This is indicated, on the one hand, by the fact that those receiving more vitamin G and growing more rapidly showed evidence of superior adult vitality as far into middle life as these experiments were continued; and, furthermore, their young showed superior vitality both in their growth rates before and after weaning and in their ability to cope with the emergency of being transferred to a vitamin G-deficient diet.

These findings make clear that the optimal intake of vitamin G is much above the amount which is easily demonstrable as strictly necessary. It is probable that the true optimum is higher than the highest level here fed.

We hope to extend this work by including studies of vitamin G, perhaps in the form of concentrates as well as in natural forms, in the investigation of the relation of food to length of life which is now being carried on with aid granted by the Carnegie Corporation of New York through the Carnegie Institution of Washington.

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## THE EFFECT OF DILUTE ALKALI ON THE CYSTINE CONTENT OF CASEIN\*

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A characteristic but insufficiently recognized property of casein is the ease with which it is acted upon by chemical reagents. Several investigators have noted that casein is more readily acted upon than most other proteins. Chittenden and Maera (1) found that when casein is heated at 100° with acidulated water, caseoses and peptones are formed. Work recently done in this Bureau<sup>1</sup> showed that after 1 hour's digestion *in vitro* with pepsin, casein was broken down practically completely into products of a peptone-like nature, about one-third of which was almost free from cystine, but which contained nearly all the phosphorus. Rettger (2) showed that when normal milk is heated above 85°, a partial decomposition occurs, with liberation of a volatile sulfide, probably hydrogen sulfide. The casein of the milk was found to be the prominent source of the sulfur liberated. When commercial casein was dissolved in dilute potassium hydroxide and precipitated by acidification with a small quantity of acid potassium phosphate, and the product was heated in a boiling water bath, hydrogen sulfide was readily given off. Casein is particularly susceptible to changes produced by the action of alkali.

A method commonly used for the purification and preparation of casein for scientific purposes consists in dissolving the casein in dilute sodium hydroxide, or potassium hydroxide, and precipitating the protein from the alkaline solution by addition of acid. Some, if not most, of the casein sold as high grade casein for scien-

\* Acknowledgment is made of the technical assistance of Mr. S. Phillips in this study.

<sup>1</sup> The results of this work will be published later in detail.

tific work, such as Hammarsten C.P. casein, has been subjected to the action of alkali during the course of its preparation.

The ease with which casein is affected by different reagents raises the question whether alkali as commonly used in the preparation of pure casein may not produce changes in its properties and composition which make the product unreliable for use in many scientific experiments. In experimental feeding tests with small animals casein is generally used to supply the protein in the basal rations. It is generally recognized that casein is a "complete" protein and that when fed at adequate levels in the diet it supplies sufficient quantities of the amino acids indispensable for the nutrition of animals. If a method used in preparing the casein reduces its available content of any one of these amino acids to a point inadequate to meet the nutritional requirements of an animal, the casein then becomes a limiting factor in all determinations and assays in which the rate of growth of animals is taken as a measure of the unknown substance.

It is well known that cystine, one of the recognized nutritionally essential amino acids, is readily decomposed by the action of alkalis. In a recent article by Thor and Gortner (3) a bibliography is given covering some of the most important work done on the action of alkali on cystine. Thor and Gortner found, confirming the earlier work of Gortner and Sinclair (4), that even dilute solutions of sodium carbonate are much more effective in removing sulfur and nitrogen from the cystine molecule than strong (20 per cent) solutions of sodium hydroxide or potassium hydroxide. Andrews (5) found that in the decomposition of cystine by alkali the production of ammonia is inversely proportional to the concentration of alkali from 4.0 M to about 0.2 M sodium hydroxide. These data emphasize the danger attending the use of even very dilute solutions in the preparation of casein.

In view of the fact that casein at the best has a relatively low content of cystine, even the destruction of a little of this amino acid during the preparation of the protein could easily reduce the quantity below the minimum requirements at the levels usually incorporated in experimental rations. Sullivan (6) found variations in the cystine content of casein from different sources ranging from 0.15 to 0.50 per cent, and he pointed out that the discrepancies found in the literature in the percentages of cystine in casein

may be due to the effect of the different methods by which it was prepared. He suggested that in feeding experiments only casein of a known cystine content should be used.

A consideration of the evidence gathered from various sources made it appear very probable that the use of alkali as commonly practiced in the preparation of what is considered high grade casein may produce far reaching effects upon its properties and composition. The extensive use of such purified casein in many different fields of scientific research suggested the desirability of obtaining definite information on this question. Accordingly, a casein preparation of high degree of purity was made in the laboratory at low temperature by a method in which the use of alkali is entirely avoided. This preparation was then successively precipitated from dilute alkali solution, and the composition of the product after each precipitation was compared with that of the original casein. The effect of the treatment was most marked upon the cystine. Its content was reduced by each precipitation, until after the fifth only a trace remained. Other changes in the composition of the casein were produced in a lesser degree.

#### EXPERIMENTAL

The casein used, which was prepared according to the method of Van Slyke and Baker (7), was of a high degree of purity. It had the following percentage composition: ash, 0.11; moisture, 9.05; nitrogen (calculated on an ash- and moisture-free basis), 16.11; cystine, 0.331 (determined by Folin and Marenzi's method (8)) and 0.336 (determined by Sullivan's method). A large number of preparations made at various times by this method have agreed very closely in their composition.

150 gm. of the casein, slightly moistened with alcohol, was suspended in 2 liters of distilled water. 25 cc. of 25 per cent sodium hydroxide solution were slowly added, and the mixture was stirred for 20 to 30 minutes, the temperature being kept at 16-20°. At the end of this time all the casein was dissolved. The solution was brought to pH 4.5 to 4.6 by slowly adding dilute acetic acid (13 per cent). The precipitated protein was allowed to settle and the supernatant liquid decanted. The residue was stirred thoroughly in about 4 liters of dilute acetic acid having the same pH as that at which the casein was originally precipitated. This process of

washing was repeated four times. After the last decantation the protein was separated by centrifugation, treated with alcohol and ether in the usual way, and dried in an oven at 110°. The protein was then exposed in a shallow layer to the air for 48 hours in order to allow it to reach moisture equilibrium so as to facilitate accurate weighing of samples for analyses. Aliquots were taken for moisture, ash, nitrogen, and cystine determinations.

The protein was then dissolved in sodium hydroxide, reprecipitated, dried, and analyzed in the same manner as described. This process of reprecipitation from dilute alkali was repeated four times. After each precipitation the dried product was analyzed for moisture, ash, and cystine. Nitrogen was determined in the

TABLE I

*Effect of Repeated Precipitations from Dilute Sodium Hydroxide Solutions upon Cystine Content of Casein*

Preparation	Cystine*
	<i>per cent</i>
Original casein.....	0.336
Product once precipitated .....	0.237
“ twice “ .....	0.199
“ three times precipitated.....	0.165
“ four “ .....	0.093
“ five “ .....	0.033

\* Percentages corrected for ash and moisture.

product only after the fifth precipitation. The cystine determinations were made according to the method of Sullivan (6).

The percentages given in Table I show that each precipitation of the protein from the sodium hydroxide solution was attended with a marked loss of cystine. Each precipitation, with the exception of the first, involved a higher percentage loss than the one before it, the final product containing only one-tenth of the cystine present in the original casein.

In view of the drastic effect produced by the alkali-acid treatment of casein, it was considered of interest and importance to know the nature and extent of other changes produced in the composition of the casein by the same treatment. Accordingly, samples of the original casein and of the protein obtained after

five precipitations from sodium hydroxide solution as described were analyzed by the Van Slyke method for distribution of nitrogen (Table II).

The percentages of arginine, histidine, and lysine given in Table III were calculated from the results obtained by the Van Slyke method. Cystine was determined by Sullivan's method, and tyrosine and tryptophane were determined, respectively, by the method of Folin and Ciocalteu (9) and by that of May and Rose (10), as modified by the authors and Moeller (11).

TABLE II

*Distribution of Nitrogen in Original Casein, and in the Product after Five Precipitations from Dilute Sodium Hydroxide Solutions\**

	Original casein (duplicate samples of 3.0 gm. each (ash- and moisture-free) contain- ing 0.4833 gm. N)		Precipitation product (duplicate samples of 3.0 gm. each (ash- and moisture-free) contain- ing 0.5055 gm. N)	
	gm.	per cent	gm.	per cent
Amide N.....	0.0557	11.53	0.0589	11.65
Humins adsorbed by lime.....	0.0050	1.04	0.0046	0.91
" in ether-amyl alcohol extract.....	0.0011	0.23	0.0018	0.36
Cystine N.....	0.0043	0.89	0.0030	0.60
Arginine ".....	0.0561	11.60	0.0536	10.60
Histidine ".....	0.0296	6.12	0.0312	6.17
Lysine ".....	0.0298	6.17	0.0491	9.71
Amino " of filtrate.....	0.2889	59.77	0.2635	52.13
Non-amino N of filtrate.....	0.0127	2.63	0.0353	6.98
Total regained.....	0.4832	99.98	0.5010	99.11

\* The figures represent average results of closely agreeing duplicate determinations.

In general, these results show that, with the exception of cystine, the amino acids of casein are not greatly affected by the alkali treatment. The greatest variation was noted in the lysine content. It is believed, however, that not much significance should be given to this difference. The estimation of lysine by the Van Slyke method is indirect, and the higher figure for lysine in the alkali-treated protein may include other forms of nitrogen resulting from the effect of the alkali upon the casein.

There can be little doubt that the decrease in the cystine content

of casein after precipitation from dilute alkali can be attributed to the destruction of cystine, and not to the mere removal of impurities from the original casein. The method by which the original casein was prepared and its extensive washings practically preclude the presence of any significant quantity of lactalbumin or of alcohol-soluble protein. The progressive decreases in the cystine values on repeated precipitation until the final product contained only a trace can be explained only on the ground of chemical action produced upon the casein molecule.

Without doubt many discrepancies in the results of scientific work, in which so called purified casein was used, can be explained on the ground that this protein did not represent unchanged casein,

TABLE III

*Nitrogen and Amino Acids in Original Casein, and in the Product after Five Precipitations from Dilute Sodium Hydroxide Solutions\**

	Untreated casein	Alkali-treated casein
	<i>per cent</i>	<i>per cent</i>
Nitrogen	16 11	16 85
Cystine	0 34	0 03
Arginine	5 81	5 55
Histidine	3 64	3 84
Lysine	5 18	8 54
Tryptophane	2 23	2 60
Tyrosine	7 49	7 63

\* Percentages corrected for moisture and ash

but was an altered protein produced by the method used in its preparation. Various workers have called attention to the unsatisfactory growth of animals in which the protein was supplied by casein which had been subjected to certain treatments. Coward, Key, Morgan, and Cambden (12) observed marked differences in the growth of rats fed rations containing different casein preparations. The animals ceased to grow when fed a vitamin A-free ration, in which Glaxo vitamin-free casein supplied the protein, but resumed growth when the Glaxo casein was substituted in the ration by another commercial preparation, light white casein (British Drug Houses). The differences noted in the nutritive properties of the two casein preparations were attributed to

the presence in the light white casein of some unrecognized growth-promoting factor not present in the Glaxo casein. The reason that the rats did not grow on the Glaxo casein ration, but grew on the light white casein, can well be explained on the ground of cystine deficiency in the Glaxo casein. That this was actually the case is made highly probable by results recently published by Prunty (13). In connection with a study on methods for the determination of cystine in biological material, he determined cystine in a number of commercial casein preparations. Light white casein was found to contain 0.32 per cent cystine, a figure which is regarded as close to the actual value for casein. In Glaxo casein, on the other hand, only 0.11 per cent of cystine was found, a value so low that the failure of rats to grow was inevitable when they were fed the Glaxo casein ration of Coward and co-workers. The following statement of Coward, Key, Morgan, and Cambden, regarding the striking difference in the rate of growth of rats in their experiments when the two different casein preparations were used in the basal rations, illustrates the importance of first establishing the adequacy of the protein used in basal rations, and shows how entirely wrong conclusions can be drawn from the results of nutritional experiments in which the quality of the protein used was unsatisfactory:

"This experiment clearly demonstrates that any dose of cod liver oil can bring about very different responses in the growth of rats according to the kind of casein which is their source of protein. In the experiment described in this paper, the sample of cod liver oil would have been considered a rich source of vitamin A if the test had been carried out on the diet containing casein B. On the other hand, it would have been considered a poor source of vitamin A if it had been carried out on a diet containing casein G."

Nutritional differences between Glaxo casein and light white casein have more recently been noted by Mapson (14). Young rats fed a basal ration in which Glaxo casein supplied the protein failed to grow and developed symptoms of nutritional deficiency. Substitution of light white casein in the basal ration for the Glaxo casein resulted in complete recovery of the animals. The failure of the animals to grow on the Glaxo casein ration is ascribed to a deficiency of a hitherto unrecognized growth-promoting factor which has been provisionally named *physin*. Even if all the nutritional deficiency features in the



experiments described by Mapson cannot be satisfactorily explained on the ground of cystine deficiency alone, cystine should be given serious consideration as a contributing factor.

#### SUMMARY

Casein is more readily acted upon by chemical reagents, particularly by alkalis, than most proteins. A casein preparation of high degree of purity was successively precipitated by acid from dilute sodium hydroxide solution after the manner usually followed in preparing pure casein for scientific purposes. The composition of the product after each precipitation was compared with that of the original casein. The most pronounced change was found in the cystine content, which was reduced by each precipitation, the product after the fifth precipitation containing only one-tenth of that present in the untreated casein. Attention is called to the dangers involved in the use of so called purified casein, both in chemical work and in experimental feeding experiments in which casein is used to supply the protein in basal rations.

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## THE NATURE AND AMOUNT OF NON-DIFFUSIBLE CALCIUM IN PROTEIN SOLS

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### INTRODUCTION

Many different workers have subjected blood serum to very careful ultrafiltration and dialysis experiments. As a result of these experiments, it appears that a part of the serum calcium is combined with the protein or some other component of the sol as an un-ionized complex which is non-diffusible through collodion or similar membranes. It further appears that the other inorganic ions which are normally present in appreciable amounts in the blood either do not undergo this type of combination at all, or undergo it to nearly the same extent.

That the non-diffusible or protein-bound calcium is in equilibrium with the calcium ions remaining in solution as such is immediately suggested by the results of Loeb (1) who found that serum calcium is completely diffusible in the presence of a large volume of 0.8 per cent sodium chloride. Other workers have concluded from solubility data that serum is or may be supersaturated with calcium phosphate. Under such conditions, the possibility of the existence of non-diffusible calcium as colloidal calcium phosphate must be admitted.

Electromotive force methods, while possessing many attractive features, have been found unsatisfactory on account of the difficulty experienced in finding an electrode which is reversible to calcium ions.

The present work was undertaken in an effort to apply the calcium electrode of Corten and Esterman (2) to the measurement of calcium ion activities in protein sols. The sols used in this work were made up of ash-free gelatin in solutions containing only po-

tassium chloride and calcium acetate. Thus the possibility of forming colloidal calcium phosphate or carbonate was eliminated. Dialysis experiments were also carried out on similar sols in order to compare the results obtained by the two different methods.

### *Materials*

*Water*—Redistilled conductivity water was used for all solutions.

*Inorganic Salts*—All salts were of c.p. grade and were recrystallized two or more times from conductivity water.

*Calcium and Zinc Oxalates*—The insoluble oxalates were precipitated from solution in conductivity water with purified ammonium oxalate and recrystallized calcium and zinc salts. The precipitated oxalates were washed repeatedly with water and finally dried at 110°.

*Zinc Amalgam*—Zinc amalgam was prepared by the electrolysis of pure zinc sulfate solution with a mercury cathode. The same amalgam was used throughout the series of measurements.

*Gelatin*—Eastman's best grade of ash-free isoelectric gelatin was used without further treatment. It was never heated above 50° in making up the sols.

*Dialysis Membranes*—Cellophane "sausage skin" dialyzers were washed thoroughly with conductivity water and dried in air before being used.

### *Electrometric Measurements*

The calcium electrodes used in these measurements consisted of approximately 10 per cent zinc amalgam in contact with an intimate mixture of solid calcium and zinc oxalates, the electrode solution being 1.0 N with respect to potassium chloride and containing a known concentration of calcium salt. This is the electrode of Corten and Esterman (2) except for the substitution of potassium chloride for potassium nitrate in the electrode solution. Preliminary experiments showed that this substitution had no undesirable effect on the electrode, and the change was made in order to reduce the diffusion potential in our measurements and at the same time to avoid any possible effect of the high concentration of nitrate ions on the protein material. Calcium acetate was used rather than some other soluble calcium salt for its buffer effect in the solution. These sols as well as those used in the

dialysis experiments were tested with suitable indicators both before and after the measurements were made. The pH of the sols was approximately 7.2 and in no case was there an appreciable variation from this value. The electrodes were made up in the type of half-cell commonly used for calomel electrodes, and all measurements were made in a thermostat at 25°.

Electromotive force measurements were made by combining the calcium electrode with a 1 N calomel electrode with N potassium chloride in the intermediate vessel. Check determinations with different calcium electrodes agreed in general to within 0.3 millivolt. The recorded values are the mean values obtained by

TABLE I

*Effect of Gelatin on Activity of Calcium Ions in Solution As Calculated from Electromotive Force Data*

CaAc <sub>2</sub>	-E' 0.5 per cent gelatin	-E 0.0 per cent gelatin	$\alpha'/\alpha$	C'Ca <sup>++</sup> 0.5 per cent gelatin	CaAc <sub>2</sub> - C'Ca <sup>++</sup> 0.5 per cent gelatin	Per cent decrease 0.5 per cent gelatin
<i>mole per l.</i>	<i>volts</i>	<i>volts</i>		<i>mole per l.</i>	<i>mole per l.</i>	
0.0025	1.1237	1.1165	0.566	0.00141	0.00109	43.4
0.0050	1.1172	1.1110	0.612	0.00306	0.00194	38.8
0.0100	1.1129	1.1070	0.627	0.00627	0.00373	37.3
0.0200	1.1064	1.1005	0.627	0.01254	0.00746	37.3
0.0300	1.1023	1.0976	0.689	0.02067	0.00933	31.1
0.0500	1.0960	1.0931	0.795	0.03975	0.01025	20.5
0.1500	1.0864	1.0851	0.902	0.1353	0.0147	9.8
0.2000	1.0857	1.0847	0.924	0.1848	0.0152	7.6

use of at least six different calcium electrodes of the same composition. The results of a series of measurements with different concentrations of calcium acetate are given in Table I.  $E$  is the voltage of the half-cell containing no gelatin measured against the normal calomel electrode, and the corresponding value for the half-cell containing 0.5 gm. of gelatin per 100 cc. of solution is  $E'$ . The corresponding activities of calcium ions in the solutions are designated as  $\alpha$  and  $\alpha'$ , and the activity ratio is calculated by use of the relation

$$E' - E = RT/2F \ln \alpha'/\alpha \quad (1)$$

We may write the activity ratio as follows:

$$\alpha'/\alpha = \gamma'C'/\gamma C \quad (2)$$

where  $\gamma'$  and  $C'$  are the activity coefficient and concentration of calcium ions in the solution containing gelatin and  $\gamma$  and  $C$  are the corresponding quantities for the non-gelatin solution. In the absence of definite knowledge of the activity coefficients of ions in protein sols, we may make the reasonable assumption that the activity coefficient of the calcium ion is not appreciably affected by the presence of a small amount of gelatin. Thus the activity ratio becomes equal to the concentration ratio, and if we assume that the concentration of calcium ions in the non-protein solution is equal to the concentration of calcium acetate, we may write for the protein sol

$$C'_{Ca^{++}} = \alpha'/\alpha \times C_{CaAc_2} \quad (3)$$

The values in the last two columns of Table I should not be taken as the amounts of protein-bound calcium per liter of sol. The concentration of ions as calculated from electromotive force data is the number of moles of ions per liter of dispersion medium. The number of moles of calcium ions per liter of sol is therefore  $C'_{Ca^{++}} \times (1 - \Phi)$ , where  $\Phi$  is the fraction of the total sol volume which is occupied by the disperse phase. This correction will be taken up more fully in the discussion of dialysis methods.

### *Dialysis Experiments*

In these experiments, a water solution containing approximately 0.08 mole of sodium chloride and 0.005 mole of calcium acetate per liter was separated by a cellophane membrane from an identical solution to which gelatin had been added. After equilibrium was attained, the two solutions were removed and carefully analyzed for chloride and total calcium. Owing to the high viscosity of the gelatin sols, all samples for analysis were measured out at 45°. Chloride was determined by the method of Van Slyke (3) as modified by Wilson and Ball (4). Calcium was determined by the procedure recommended by Kramer and Tisdall (5). The accuracy claimed by the authors for this method was greatly improved by increasing the amount of calcium in the samples being analyzed.

Table II contains the results of a series of dialysis experiments. The first five columns contain the analytical data. The results in the last three columns were calculated in the usual way. It was

assumed that the analytical data give directly the chloride ion concentration in both solutions, the calcium ion concentration in the non-protein solution, and the total calcium in the protein sol. The concentration of calcium ions in the protein sol was calculated by means of the following expression of the Donnan equilibrium:

$$(\text{Cl}^-_{\text{aq.}}/\text{Cl}^-_{\text{sol}})^2 = \text{Ca}^{++}_{\text{sol}}/\text{Ca}^{++}_{\text{aq.}} \quad (4)$$

or

$$\text{Ca}^{++}_{\text{sol}} = (\text{Cl}^-_{\text{aq.}}/\text{Cl}^-_{\text{sol}})^2 \times \text{Ca}^{++}_{\text{aq.}} \quad (5)$$

In this equation, the assumption is again made that the activity coefficients of the ions are not affected by the presence of the gela-

TABLE II

*Effect of Gelatin Concentration on Relative Amounts of Diffusible and Non-Diffusible Calcium As Calculated by the Usual Method*

The figures are expressed in moles per liter.

Per cent gelatin	$\text{Cl}^-_{\text{aq.}}$	$\text{Cl}^-_{\text{sol}}$	$\text{Ca}^{++}_{\text{aq.}}$	$\text{Ca}_{\text{sol}}$ (total)	$\text{Ca}^{++}_{\text{sol}}$	Bound Ca	Per cent bound
0.5	0.0815	0.0798	0.00516	0.00555	0.00538	0.00017	3.1
1.0	0.0817	0.0806	0.00513	0.00563	0.00527	0.00036	6.4
1.5	0.0812	0.0790	0.00546	0.00629	0.00577	0.00052	8.3
2.0	0.0817	0.0801	0.00484	0.00589	0.00503	0.00086	14.6
2.5	0.0828	0.0802	0.00506	0.00617	0.00539	0.00078	12.6
3.0	0.0813	0.0804	0.00477	0.00587	0.00488	0.00099	16.9
3.5	0.0817	0.0802	0.00460	0.00576	0.00477	0.00099	17.2
4.0	0.0815	0.0801	0.00466	0.00587	0.00483	0.00104	17.7

tin. The amount of bound calcium in the sol was determined by difference. Increasing concentrations of gelatin rather than of calcium acetate were used in this series of experiments in order to increase the relative amount of combined calcium and to improve correspondingly the accuracy of our calculated values.

#### DISCUSSION

Comparison of the values in the last three columns of Table II with the corresponding values of Table I shows that the concentration of calcium ions in the protein sol as calculated from the results of dialysis experiments is much greater than the values calculated from electrometric measurements under similar conditions. This discrepancy is due to an error that has been made in

the application of the Donnan equilibrium to the calculation of the results of dialysis and ultrafiltration experiments. The error is due to the failure properly to correct for the fact that the concentration terms in Equation 4 refer not to the number of moles of ions per liter of protein sol but to the number of moles per liter of dispersion medium. Thus, the effective concentration of a solute ion in the sol is not the analytically determined concentration,  $C$ , but  $C/(1 - \Phi)$ , where  $\Phi$  is the volume of protein hydrate disperse phase per unit volume of sol. Introducing this correction into the ion concentration terms in the sol in Equation 4, and solving for the number of moles of calcium ions per liter of sol, we obtain

$$\text{Ca}^{++}_{\text{sol}} = (1 - \phi)^2 (\text{Cl}^{-}_{\text{aq}} / \text{Cl}^{-}_{\text{sol}})^2 \times \text{Ca}^{++}_{\text{aq}} \quad (6)$$

Several different methods are available for determining the value of  $\Phi$ . The viscosity equation of Einstein (6) probably gives satisfactory values from a physical standpoint, but the values obtained sometimes seem unreasonably large when compared with the results of other methods. This is probably due to the fact that the Einstein equation measures the volume of the protein particles plus all of the water which is carried with them in their kinetic motion. Other methods, notably those based on osmotic pressure and solubility measurements, measure, in effect, the solvent power of the sol as compared with that of an equal volume of pure water. Thus, the value of  $\Phi$  as determined by the Einstein equation probably includes a certain volume of hydrate water which is loosely held by the protein particles but retains at least a portion of its solvent power and is therefore only partially included in the  $\Phi$  values calculated from osmotic pressure and other measurements. Probably the most convenient and satisfactory method for determining  $\Phi$  for use in Equation 6 is by means of the empirical viscosity equation of Kunitz (7) (Equation 7) who has shown a remarkable correlation of the results obtained by means of this equa-

$$\eta = (1 + 0.5\phi)/(1 - \phi)^4 \quad (7)$$

tion and the results of his osmotic pressure measurements. It should be kept in mind, however, that viscosity equations are apt to give abnormally high values for  $\Phi$  if the electrokinetic potential of the particles is high. This error is probably small in the pres-

ence of a few hundredths  $M$  or higher concentration of sodium chloride or other soluble salt, and for isoelectric protein in pure water. Calculations of  $\Phi$  based on the partial specific volume as determined by density measurements are meaningless for highly hydrated proteins because no account is taken of hydration and the resulting increase in the volume of the protein phase at the expense of the continuous phase.

The value of  $\Phi$  for normal blood serum as determined by Equation 7 is about 0.15, and therefore the previously calculated values of the amount of calcium ion in serum will be reduced by about 40 per cent, with a corresponding increase in the amount of combined calcium. We have not attempted to apply the correction to our data on gelatin sols on account of the unreliability of viscosity measurements made near the gelation temperature.

Quantitative comparison of the results of the two methods is also impossible because of the fact that in no case was exactly the same system studied by both methods. However, the system represented by the second line of figures in Table I is quite similar to that represented by the first line in Table II. It is interesting to note that quantitative agreement of the results of the two methods is obtained if the data are recalculated with the not unreasonable value of  $\Phi = 0.2$  for both of these systems. On the other hand, if the  $\Phi$  value is less for the higher concentration of alkali chloride used in the electrometric measurements, quantitative agreement requires a slightly lower  $\Phi$  value for the other system also.

The manner in which the amount of protein-bound calcium increases with increasing calcium ion concentration and with increasing protein content of the sol suggests either a specific adsorption process or the formation of a partially ionized calcium-gelatin salt. In either case the chemical properties of the calcium ion would be expected to play an important part in the process. On the other hand, the protein particle is a negative colloidal particle surrounded by an atmosphere of positive ions. Under such conditions, the tendency is for positive ions of high valence to enter the ionic atmosphere to a greater extent than ions of lower valence. Thus the calcium ion having the same average kinetic energy as a univalent ion but twice the attraction for the protein particle would tend to displace univalent ions and remain



more closely held by the colloidal particle. If this valence effect is of importance, we would expect other bivalent ions if present to undergo this same type of "combination" or non-specific adsorption. This is apparently true for the ions of magnesium (8) and zinc (9).

#### SUMMARY

It has been shown by both electrometric and dialysis methods that the non-diffusible calcium in protein sols is present as a calcium-protein complex and not as a colloidal inorganic precipitate.

Published values for the amount of combined calcium in protein sols are in error owing to failure properly to correct for the volume of the disperse phase. Making the proper correction will lead to somewhat higher values for combined calcium.

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## A METHOD FOR THE QUANTITATIVE ESTIMATION OF INDOXYL COMPOUNDS IN BLOOD

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The procedure given below for the quantitative determination of indoxyl compounds in blood differs essentially in but one respect from that recently presented by the writer for the estimation of indoxyl compounds in urine (1). For bloods containing any but exceedingly high concentrations of indoxyl, all reactions must be carried out at room temperature. The use of a boiling water bath as indicated for the determination in urine gives small but definite increments in color formation due to substances other than indoxyl. While solutions of indican give identical values with both the urine and blood methods upon use of a boiling water temperature, neither method is quantitative with amounts of indican in excess of 0.015 mg. when carried out at room temperature. For this reason, to effect what would be essentially both quantitative and specific results, in our procedure we must employ room or boiling water temperature as dictated by the absolute amount of indican in the blood sample tested. A practical guide to which procedure is to be used in any one sample is given below.

### *Determination of Indoxyl Compounds in Blood*

The estimations are made on blood plasma, potassium oxalate or sodium citrate being used as an anticoagulant in the collection of the blood.

*Reagent A*—Protein coagulant, made by adding to each 25 gm. of trichloroacetic acid 100 cc. of distilled water.

*Reagent B*—1 per cent potassium persulfate in distilled water.

*Reagent C*—1 per cent thymol in 95 per cent ethyl alcohol.

*Reagent D*—Acid reagent, made by adding to each 12.5 gm. of trichloroacetic acid 100 cc. of concentrated hydrochloric acid (c.p.).

*Reagent E*—Glacial acetic acid.

*Procedure*—Place 2 cc. of blood plasma in a test-tube and dilute to 3 cc. with distilled water. Add from a burette 5 cc. of Reagent A after the following manner: three successive additions of 5 drops, each addition followed by vigorous stirring with a glass rod for 15 seconds. This detail is most essential. It effects a fine and quite complete protein precipitation, resulting in a satisfactorily clear filtrate and maximum adsorption of the acid by the protein particles. The balance of Reagent A is added in two approximately equal portions with stirring after each. The tube is then centrifuged at moderately high speed for 10 minutes. The filtrate is then delivered, through a 5.5 cm. quantitative filter paper, into a 15 cc. centrifuge test-tube graduated in 0.1 cc.

To 6 cc. of the filtrate are added successively 5 drops of Reagent B, 0.5 cc. of Reagent C, and 5 cc. of Reagent D. The tube is then momentarily corked and inverted and then allowed to stand at room temperature for a minimum of 50 minutes. Within 5 minutes after the last reagent is added, a dense white cloud forms in the mixture (ethyl trichloroacetate). In about 15 minutes or sooner, if approximately 0.015 mg. of indican is present in the mixture, this cloud takes on an unmistakable purple tinge. When this occurs, it is necessary to resort to the use of the boiling water bath completely to release all the indoxyl for a quantitative production of the pigment condensate (indoxyl-thymol compound). In this event, the tube is at any convenient time placed in a boiling water bath and kept there for 12 minutes. If no distinct purple color develops within 15 minutes, heating is unnecessary.

At the end of the 50 minutes at room temperature, or after the use of the water bath, when needed, the tube is centrifuged at high speed for 10 minutes, completely to throw down the ethyl trichloroacetate and the indoxyl-thymol compound contained therein. The volume of the ester formed is approximately 0.25 cc. with the determination carried out at room temperature and about 0.6 cc. when the water bath is used. The supernatant fluid is then pipetted off, leaving a maximum of 0.3 cc. of overlying watery acid mixture. This mixture of acetate and acid water is diluted to exactly 2 cc. with Reagent E delivered from a burette. On stirring, a perfectly transparent colored solution results. Occasionally a fine white film is noted on the surface of the acetate but this too dissolves in Reagent E. The solution is now ready for

reading in a microcolorimeter, through a green filter (Wrattan No. 74) inserted in the eye-piece, as described for the urine method.

For a standard a 0.75 per cent solution of  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  (Merck, C.P.) is used. Its indican equivalent (0.0043 mg.) is identical with that determined for its use in the method for urine analysis. This follows from the fact that indican solutions, analyzed both by blood and urine methods, give identical chromogenic values.<sup>1</sup> A corroboration of this is found in the satisfactory recovery of added indican to blood with the added indican determined by the method for urine analysis.

With blood as with urine, omission of the thymol reagent (substituting therefore an equivalent volume of 95 per cent ethyl alcohol for the formation of the ethyl trichloroacetate) prevents the production of any pigment condensate with indoxyl. Since ethyl trichloroacetate might readily carry down with it anything in blood soluble in an organic solvent, lipids especially, as well as the indoxyl-thymol pigment, determinations on bloods were carried through with thymol omitted. These disclosed that the necessary correction was greater than in the determination of indican in urine. In the latter, allowance must be made only for the absorption of light by the ethyl trichloroacetate. In the determination of indican in blood, other substances extracted from the reaction mixture are of greater significance. The correction necessary is 0.123 mg. per 100 cc. of blood plasma.

The method of calculation for blood indican follows.

$$\begin{aligned} &\text{Mg. indican per 100 cc. blood plasma} \\ = &\frac{0.0043 \times \text{reading of standard (mm.)}}{\text{reading of unknown (mm.)}} \times \frac{100}{15} - 0.123 \end{aligned}$$

In Table I are given data covering indican determinations in blood samples before and after the addition to each of pure indican. Added indican was colorimetrically determined by the method for

<sup>1</sup> The use of the blood method on indican solutions requires a combining of the protein-coagulating and acid reagents in proper proportion as a single mixture to be added as the last reagent. Strong trichloroacetic acid solutions on standing with pure indican solution for 20 minutes destroy up to 25 per cent of the indican. In the presence of protein this destruction does not occur. The explanation may be that the protein coagulum absorbs enough acid to inhibit such destruction.

urine analysis. The recoveries were quite satisfactory. It should be pointed out that satisfactory recoveries of added indican colorimetrically determined could only be achieved if, in addition to a technical procedure permitting such recoveries, the values of the

TABLE I  
*Indoxyl (As Indican) Determination on Addition of Indican to Blood*

Sample No.	Indican found per 1.5 cc. plasma	Indican added per 1.5 cc. plasma*	Total indican determined	Percentage of calculated total
1	0.0002	0.0061	0.0057	91
2	0.0011	0.0046	0.0061	107
3	0.0010	0.0081	0.0084	92
4	0.0021	0.0137	0.0145	92
5	0.0030	0.0035	0.0068	104
6	0.0007	0.0047	0.0054	100
7	0.0009	0.0134	0.0131	92
8	0.0008	0.0061	0.0068	99
9	0.0018	0.0072	0.0089	99
10	0.0003	0.0049	0.0048	92
11	0.0002	0.0082	0.0077	92
12	0.0062	0.0086	0.0149	100

\* Colorimetrically determined by method for urine analysis.

TABLE II  
*Indican Determination in Blood from Apparently Normal Individuals Whose  
Total Non-Protein Nitrogen Was Less Than 35 Mg. Per Cent*  
Blood samples were taken before breakfast.

Sample No.	Indican per 100 cc. plasma	Sample No.	Indican per 100 cc. plasma
	mg.		mg.
1	0.002	8	Blank
2	Blank	9	0.013
3	0.039	10	Blank
4	0.048	11	0.046
5	0.024	12	0.044
6	0.020	13	0.054
7	0.002		

corrections employed in the two determinations were substantially correct.

In Table II are given indican determinations in apparently normal individuals in each of whom the total non-protein nitrogen

was less than 35 mg. per cent. In 100 cc. of plasma there is less than 0.06 mg. of indican; not infrequently the values are blank. By the latter we mean that in making our calculations from the readings the value subtracted as a correction leaves zero or a minus quantity as the final measure. An almost immeasurably small concentration of indican, then, exists in the blood of normal fasting individuals. On the other hand, with nitrogen retention, the concentration of indican in the blood may rise to more than 100 times the maximum in normal blood, as may be seen from Table III.

TABLE III  
*Determinations on Bloods Showing Exceedingly High Concentrations of Indican*

Case No.	Non-protein N	Indican	Diagnosis
	<i>mg. per cent</i>	<i>mg. per cent</i>	
1	120	5.70	Hypertrophied prostate
2	85	2.00	Cardiac disease, ascites
3	120	4.50	Hypertrophied prostate
4	150	1.55	Bladder stone
5	200	2.46	Acute nephritis
6	75	1.50	Nephritis
7	110	1.86	Hypertrophied prostate
8	85	2.05	Cardiorenal disease
9	250	6.20	Removal of only kidney*
10	85	1.84	Nephritis

\* Removed for traumatic rupture; autopsy revealed congenital absence of a second kidney.

### *Comment*

No other method has as yet been proposed for the quantitative measurement of indican on reasonably small quantities of normal blood.<sup>2</sup> Haas (3) who adapted Jolles' method (4) to blood, used 40 cc. of serum to determine normal concentrations. This he found to be between 0.02 and 0.08 mg. per cent. These estimations were in conformity with our findings.

<sup>2</sup> The micromethod sketchily outlined by Rappaport and Engelberg (2) could hardly be expected to measure indican in normal blood; nor could a waiting period of 12 hours be recommended as a step in a practicable method.

It is evident that we cannot claim specificity for our method for blood indican since it involves the measurement of variables definitely not indoxyl—substances extracted by ethyl trichloroacetate with measurable influence on light transmission. However, experiments have shown that these non-specific variables have a minimal and quite insignificant range while indoxyl concentrations may rise to more than 100 times the estimated normal average. For bloods with normal or moderately elevated concentrations of indoxyl, on which determinations are carried out at room temperature, the estimated correction introduced in the calculations for the non-specific variables as well as the ethyl trichloroacetate permits with reasonable certainty an interpretation of increased values above the normal as due to indoxyl.

When the use of a boiling water bath is required a non-specific and chromogenic reaction occurs. Glucose<sup>3</sup> then reacts to form a faint pink compound, soluble in the acetate. Experiments disclose that the measured value of this substance given as indican never exceeds 0.15 mg. per cent. In practise this would limit its value to a maximum of 15 per cent of the total estimated indican and so never interfere with a true clinical interpretation of the exceedingly high indican concentrations for which alone the water bath method is applied. However, when necessary, this interference can be removed by first treating the plasma with washed yeast to destroy the glucose.

#### SUMMARY

A method for the quantitative estimation of indoxyl compounds in blood is presented. Estimation of normal blood indican establishes that value as less than 0.06 mg. per cent.

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<sup>3</sup> A small fraction of the color formed is not due to glucose, since in normal blood a preliminary fermentation of the plasma with yeast does not bring the indoxyl value wholly down to the level found when the determination is completely carried out at room temperature.

## CREATINURIA AMONG ADOLESCENT MALES

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Despite many investigations, the underlying mechanism of creatinuria during the growth period still remains in doubt. That the amounts excreted fluctuate proportionately with the protein in the diet has been definitely shown by Talbott and Gamble (1), Denis and Kramer (2), and Gamble and Goldschmidt (3) on children, and by Harding and Young (4) and Harding and Gaebler (5) on puppies. Powis and Rapier (6), having found a diminished excretion during the night, conclude as follows: "The chief factor appears to be the state of rest of the skeletal muscles associated with the temporary cessation of voluntary control which occurs during sleep." Beumer and Fashold (7) believe that the capacity to utilize creatine is in some manner related to the endocrine changes of puberty.

It was suggested to us<sup>1</sup> that further information on this subject might be obtained from the study of boys of the age period at which creatinuria ceases. Many students attending the Lawrenceville School belong in this group. These boys constitute ideal subjects for such a study, having been raised with excellent care.

In this paper we wish to report a series of results obtained during the past year, from the study of 24 hour urine specimens procured from adolescent boys in this school, under various conditions, on an uncontrolled diet.

### *Methods*

A modification of the micromethod of Folin (8) was used in all our determinations, with HCl and the autoclave for the conversion

<sup>1</sup> We are indebted to Dr. Samuel Goldschmidt of the University of Pennsylvania Medical School, Department of Physiology, not only for suggesting this study, but also for his advice on many occasions.



of creatine into creatinine. Preliminary readings were made of all samples and the urines then diluted so that the final readings would come as close as possible to the standard set at 20. All samples were then run in duplicate (usually five subjects were studied on the same day). As an additional control, a known amount of pure creatine in solution was added to duplicate portions of one of these urines. These samples were then analyzed with the others. By using this additional check we found our limit of error for the total creatine to be a maximum of 10 mg. per liter of urine.

#### EXPERIMENTAL

In Table I we have summarized the results obtained by analyzing the urines of single 24 hour specimens of 81 boys. As a rule,

TABLE I

*Incidence of Creatinuria among Adolescent Males on Uncontrolled Diet, and Average Amounts Excreted (24 Hour Specimens) for Each Yearly Period*

Age period	No. of subjects studied	No. of subjects excreting creatine	Percentage excreting creatine	Average creatine excretion
<i> yrs.</i>			<i> per cent</i>	<i> mg.</i>
14-15	12	6	50	117
15-16	23	11	47.7	88.5
16-17	22	10	45.5	79.3
17-18	18	7	38.8	42.3
18-19	6	1		17

five different subjects were collecting their urines at the same time. The diet served to each group contained a normal amount of protein. Urine collections were begun following the first morning voiding. All urine was then saved through the balance of the day and night, and the first voiding of the next morning added, insuring a representative 24 hour specimen.

It will be noted from Table I that on an uncontrolled diet, among boys from 14 to 17 years of age, from 45 to 50 per cent still show the presence of creatine in the urine. Between the ages of 17 and 18 years there is a slight drop in the percentage, namely 38.8 per cent. Among six boys between 18 and 19 years of age, only one subject showed the presence of creatine. It was only on

TABLE II

*Preformed Creatinine, Creatine, Preformed and Total Coefficients of Boys  
Excreting Creatine on an Uncontrolled Diet*

Subject No.	Age period	24 hr. preformed creatinine	24 hr. creatine	Preformed creati- nine coefficient	Total creatinine coefficient
	<i>yrs.</i>	<i>mg.</i>	<i>mg.</i>		
1	14-15	1022	97	20.7	22.6
2	14-15	1258	209	27.9	32.4
3	14-15	1730	127	28.2	30.2
4	14-15	1135	52	25.0	26.1
5	14-15	1265	60	24.2	26.5
6	15-16	1465	26	23.2	23.6
7	15-16	1502	58	26.5	27.5
8	15-16	1915	183	27.7	32.0
9	15-16	1472	147	26.8	29.4
10	15-16	1735	65	26.9	27.9
11	15-16	1695	57	21.9	22.6
12	15-16	1620	15	26.4	26.6
13	15-16	1500	40	22.7	23.4
14	15-16	1080	105	21.2	23.2
15	15-16	1395	85	24.2	26.8
16	15-16	1639	193	26.6	29.8
17	16-17	1866	54	27.4	27.9
18	16-17	1503	229	25.6	29.5
19	16-17	1351	27	28.2	29.1
20	16-17	1336	86	23.3	24.7
21	16-17	1435	15	20.5	20.7
22	16-17	1453	32	26.4	27.0
23	16-17	1560	52	24.0	24.9
24	16-17	1520	75	26.1	27.4
25	16-17	1836	44	27.5	28.0
26	16-17	1668	158	27.0	29.4
27	17-18	1829	29	26.0	26.4
28	17-18	1985	99	26.9	28.2
29	17-18	1760	25	28.5	28.9
30	17-18	1540	35	24.6	25.1
31	17-18	1635	15	26.0	26.3
32	17-18	1330	38	22.3	23.0
33	17-18	1790	55	26.3	27.1
34	18-19	1566	17	23.4	23.7

\* The bold-faced figures in parentheses represent group averages for the respective age period.

rare occasions that among a group of five boys studied during the same period, that either all or none showed creatine. The average creatine excretion as shown in Table I declines for each increase in the age period.

Table II gives in detail the results obtained from the subjects who were found to show creatine in single 24 hour specimens. This table also contains the preformed and total creatinine coefficients. Although the amounts of creatine excreted show considerable variation for different boys of the same age, the average excretion as noted before and shown in Table I falls as the age period increases. On the other hand, the preformed creatinine coefficient remains remarkably constant for each yearly period.

TABLE III

*Comparison of Creatine Excretion during Rest in Bed on a High Protein Diet with a Similar Period of Normal Activity and Diet*

Subject No	Age period	Rest in bed high protein diet 7 00 a m -11 00 p m			Normal activity, normal diet 7 00 a m -11 00 p m		
		Urinary volume	Preformed creatinine	Creatine	Urinary volume	Preformed creatinine	Creatine
	yrs	cc	mg	mg	cc	mg	mg
1	16-17	810	1140	5	785	1082	114
2	15 16	1355	1050	11	520	1084	186
3	14-15	1035	1122	6	1105	1131	97
4	14-15	1325	755	10	880	741	108
5	15-16	890	896	23	695	906	95

Five subjects on an uncontrolled diet, who showed creatine in their single 24 hour specimens, were selected and placed in the infirmary for a night's rest. The following day was spent in bed, with the exception of a short walk (100 yards) to a 45 minute chapel service, where they were seated quietly. At the end of the service, they returned immediately to bed. The three meals consumed by the subjects contained large amounts of protein. The urine was collected from 7 00 a. m. until 11 00 p m.

The following morning, these boys were allowed to leave the infirmary. They immediately began a second period of collection at 7.00 a.m. and finished again at 11 00 p m. During this period they attended classes, engaged in the usual required exercise, and partook of a normal protein diet.

The results obtained by these studies are shown in Table III. It will be noted that the creatine excretion during the rest in bed period was practically negligible compared to the corresponding period of time the next day when these boys followed their normal routine.

One of our subjects on an uncontrolled diet, who had previously shown creatine in the urine, was stricken with a mild attack of grippe. Confinement to bed gave us the opportunity to study his

TABLE IV

*24 Hour Creatine Excretion on Successive Days Following Confinement to Bed Due to Illness*

The values are stated in mg.

Subject No.		Confined to bed	Limited floor privileges	Unlimited floor privileges			Nature of illness
1	Preformed creatinine	1551	1321	1430	1115	1388	Mild influenza
	Creatine	4	74	305	155	142	
2	Preformed creatinine	1357	1256	1265	1160	1211	Mild broncho-pneumonia
	Creatine	3	69	233	160	134	
3	Preformed creatinine	1018	1055	1025			Fatigue
	Creatine	212*	105	304			
4	Preformed creatinine	1638	1592	1432	1378	1539	Fractured fibula; used crutches
	Creatine	1	14	23	12	3	
5	Preformed creatinine	955	897	955	878	1042	Appendicitis
	Creatine	19	48	103	51	48	
6	Preformed creatinine		1278	1459	1260		Constipation and headache
	Creatine		93	401	309		

\* Limited floor privileges.

creatinine excretion on successive days. While he was in bed with slight fever, and on a diet containing moderate amounts of protein, his urine was found to be free of creatine. The urine collections were all made by means of the urinal and the boy kept absolutely in bed. When his temperature dropped to normal he was given a full protein diet. The urine still remained free of creatine. On the 1st day that he was allowed to be out of bed for several hours, the creatine excretion rose to 74 mg. The following day when he was allowed to be out of bed all day, creatine excretion rose to 305

mg., followed by a progressive decline for each successive day, as may be noted in Table IV, Subject 1.

With these findings at hand, five other boys were selected for similar study. In each instance, careful nursing supervision facilitated the collection of 24 hour samples for each successive day and insured the validity of the urines.

One notes in Table IV, with the exception of Subject 3, that the 1st day of limited freedom from confinement to bed is marked by a rise in the creatine excretion, followed by a decided rise the following day when unlimited floor exercise was permitted. Subject 3 was a boy 14 years of age—the youngest of this group. He was admitted to the infirmary with the complaint of feeling very tired. No evidence of infection could be found. He was given a normal diet and 24 hour urine collections were begun immediately. The results shown in Table IV cover the 3 days he spent in the infirmary. The 1st and 2nd days he spent most of the time in bed, although not required to do so. The 3rd day only 3 hours were spent in bed. The next day he was discharged. The results shown in Table IV are the results obtained throughout his 3 day stay.

The diets for each boy remained practically constant for protein during the entire study period.

#### DISCUSSION

Rose (9) was unable to find creatine in the urine of subjects on an uncontrolled diet when over 15 years of age. Harding and Gaebler (10) predicted 16 years as the age at which it would disappear from the urine in normal males on a high protein diet. Folin and Denis (11) reported its presence in the urine of a 17 year-old boy on a strictly vegetable diet.

Our results point to a wide latitude in years among adolescent males, during which creatine may be absent from a 24 hour urine specimen of an individual on a normal protein diet. What impressed us most during these studies was the frequent finding of the presence of creatine in one subject and its absence in another, both of whom were approximately the same age, weight, and height, on the same diet, and collecting their urines at the same time. Toward the end of this particular study, we were, however, further impressed with the frequency of a certain lethargy and

aversion to physical exercise among the subjects still excreting creatine.

A close relationship between creatinuria and muscular activity in adolescent males is suggested by the definitely lowered creatine excretion among a group of boys on a high protein diet while resting in bed, as compared to the amounts excreted by these same subjects the following day, when attending to their usual school routine of study and exercise and on a normal protein diet.

The marked increase in creatine excretion encountered on the 1st day of muscular activity following a period of confinement to bed, due to an illness or injury, and its progressive decrease on each succeeding day, points to the fitness of skeletal muscles to meet the demands of exercise as an additional factor in the phenomenon of creatinuria among adolescent males.

#### SUMMARY

1. Creatine was found in single 24 hour specimens among thirty-five of 81 normal boys between 14 and 19 years of age on an uncontrolled diet.

2. The percentage showing creatinuria remains fairly constant at ages from 14 to 17, drops perceptibly between 17 and 18, while it was present in only one out of six boys between 18 and 19 years of age.

3. The average creatine excretion for each yearly period declines with increasing age.

4. Five subjects on a high protein diet while resting in bed showed far less creatine excretion than on the following day on a lower protein diet while attending classes and engaging in exercise.

5. Five out of six subjects who had been confined to bed for either a minor illness or injury showed a marked increase in the creatine excretion on the 1st day of muscular activity, and a progressive decrease on each successive day.

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## FLUCTUATIONS OF THE BLOOD SUGAR IN VITRO

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In a recent paper, Kleiner and Halpern<sup>1</sup> report in one phase of their work that undialyzed hyperglycemic bloods (diabetic bloods or normal bloods fortified with dextrose) standing at room temperature frequently show marked fluctuations in their blood sugar curves. These fluctuations, it is stated, are greater than can be accounted for by analytical errors. In addition to the usual glycolytic trend seen in these curves, there is also noted evidence of sporadic glucogenesis. Such unexpected findings would seem to merit attempts at their verification.

Hyperglycemic bloods<sup>2</sup> (see Table I) were, except in a few instances, treated with novirudin<sup>3</sup> to prevent clotting. Blood sugar was determined at 20 minute intervals by the Benedict (1928) method.<sup>4</sup>

The results are recorded in Table I. No such fluctuations as described by Kleiner and Halpern occurred. Any variations from the usual glycolytic curves shown in Table I can be ascribed to the limitations of analytical methods.

<sup>1</sup> Kleiner, I. S., and Halpern, R., *J. Biol. Chem.*, **101**, 535 (1933).

<sup>2</sup> For the diabetic human bloods, I am indebted to Dr. Wyckoff, Third Medical Division, Bellevue Hospital; for the diabetic dog bloods, to Drs. Ralli and Co Tui.

<sup>3</sup> Grateful appreciation is due to Dr. I. S. Kleiner for furnishing me with the supply of novirudin used in these experiments.

<sup>4</sup> Benedict, S. R., *J. Biol. Chem.*, **76**, 457 (1928).



TABLE I  
*Mg. of Blood Sugar per 100 Cc. at Varying Intervals of Time*

Source of blood sample	Intervals at which blood sugar was determined, min.								
	0	20	40	60	80	100	120	140	180
Normal human, with added dextrose. First samples (0 min.) analyzed within 3 to 18 min. after withdrawal of blood	263*	265	258	255	252	242	238	235	
	494*	488	488	488	482	488	476	476	
	385	385	381	383	383	374	375	375	
	427	405	403	400	401	394	395		
	454	451	451	452	454	454			
	389	382	382	379	382	382	375		
	357*	354	348						
Diabetic human. First samples (0 min.) analyzed within 9 to 18 min. after withdrawal of blood	250*	245	245	242	245	242	229	230	229
	234*	226	222	221	219	217	215		
	141	137	134	131	132	125	126		
	323	312	309	308	309	303	298		
	301	296	292	294	289	288	289		
	210	209	208	208	206	203	200		
	348	351	345	345	342	345	342	339	
	247	242	235	237	237	238	238		
	296*	291	282	280	276	266	265	255	255
	308*	305	294	294	294	286	280	278	270
Normal dog, with added dextrose. First samples (0 min.) analyzed within 11 to 16 min. after withdrawal of blood	340	333	325	328	328	324	328	325	
	390	384	376	378	374	369	367		
	385	383	377	381	381	382	374		
	428	428	422	420	417	414	412		
	213*	209	201	199	197	194	189	186	
	244	237	231	231	234	230			
	296	290	284	284	284	284	287		
Diabetic dog. First samples (0 min.) analyzed within 12 to 17 min. after withdrawal of blood	282	281	272	272	269	273	272		
	312	311	309	310	311	308	308		
	336	325	328	328	328	323	328		
	488	466	468	468	470	468			

\* Anticoagulant, heparin.

# THE EFFECT OF DEHYDRATION ON THE PANCREATIC AND INTESTINAL ENZYMES

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*Enzymes in Dehydration*—The purpose of these experiments is to compare the enzyme content of the pancreas and small intestine in dehydrated and normal rats, and to relate the results to clinical conditions which are associated with dehydration.

In 1931, Banting, Gairns, Lang, and Ross (1) made an extensive study of the enzyme content of the stools of infants suffering from acute intestinal intoxication (acute fermentative diarrhea), in which one of the outstanding symptoms is dehydration. They showed that the trypsin was markedly reduced in this disease, and if daily estimations were made, the curve obtained could be used as an index of the severity of the case. The amylase and lipase of stool were also reduced, although the results obtained were not so consistently low as in the case of trypsin.

A further study was made in this laboratory (2) of the intestinal enzymes in cases dying of acute intestinal intoxication, and the crepsin and phosphatase content of the small intestine was found to be lower in this condition than in cases in which death was due to other causes.

Severe dehydration is one of the most important clinical symptoms of this condition, and invariably results from the rapid loss of fluids due to the diarrhea and vomiting. Johnston, Brown, Tisdall, and Fraser (3) have shown that in cases of acute intestinal intoxication organisms of the colon-paratyphoid-dysentery group are found in the intestinal tract, the most important member of the group being *Bacillus dysenteriae*, Sonne. In this condition also there is invariably a state of acidosis varying from a mild to a severe degree (4), and a decrease in the ability of the tissues to

tolerate injected glucose (5). The blood viscosity is greatly increased and there is a marked diminution in the blood flow in the peripheral capillaries. In acute intestinal intoxication severity of dehydration appears to be associated with a very low enzyme content of the stool. In order further to determine the association of these two conditions, it was thought desirable to produce a state of dehydration in animals, and to find what the resultant effect would be on the production of enzymes.

In these experiments 6 week-old albino rats were used. The method employed in dehydrating the animals was to place them in individual wire cages in an incubator at 37.5°. After 12 hours incubation they were removed and kept at room temperature for the succeeding 12 hours. This procedure was continued for from 3 to 4 days, depending on the vitality of the individual groups. An adequate normal diet was fed to the rats during this time, but water was withheld. Rats averaging 76 gm. in weight lost between 23 and 29 per cent of their weight after 4 days dehydration.

Although there are a number of different methods which might have been used for the determination of the enzyme content of the extracts, it was decided to follow the methods in use by the Willstätter (6) school of enzyme chemistry. These methods are better known and probably more widely used than others.

The technique for determining the amounts of the different enzymes present and the methods of calculating the units of activity are given in the experimental part. In all cases the results have been expressed in units of enzyme per gm. of tissue.

### *Pancreatic Enzymes*

The rats were killed by a blow on the head and the pancreas was dissected out, weighed, and ground with sand and a volume of 25 per cent glycerol equal to 20 times the weight of the pancreas. After 18 hours extraction at room temperature, it was centrifuged, and the tryptic and amylolytic activity of aliquot samples determined.

*Trypsin*—Trypsin is the proteolytic enzyme of the pancreatic secretion. It acts on the proteins and semidegradation products of proteins coming from the stomach, to reduce them either to assimilable amino acids or simple peptides, which are further broken down by the action of intestinal erepsin.

TABLE I

*Enzyme Content in Units per Gm. of Tissue of Normal and Dehydrated Rats*

Pancreas				Small intestine			
Trypsin		Amylase		Erepsin		Phosphatase	
Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated	Normal	Dehydrated
20 9	11 3	139	49	46 1	20 0	15 7	14 2
34 2	15 2	183	83	38 5	16 1	18 2	14 5
24 7	19 0	191	104	41 2	16 2	19 4	10 5
34 2	15 2	180	47	35 9	16 0	21 6	9 3
22 8	15 2	161	43	52 6	14 0	23 7	8 2
20 9	21 0	180	59	43 9	10 4	23 6	5 8
24 7	17 1	126	71	28 7	8 5	18 1	6 7
36 1	19 0	145	94	28 7	12 2	18 0	16 5
	11 3	134	100	22 2	14 0	12 7	3 3
	22 8	145	97	28 7	14 0	20 0	4 9
	13 3	143	95	35 9	12 2	12 5	11 5
	24 7	161	100	43 9	14 0	15 3	11 5
	20 9	139	106	35 9	20 8	13 9	8 4
	11 3	120	100	30 6	26 5	12 4	5 9
	11 3	158	102	33 3	16 1	21 3	4 7
		191	123	30 6	14 0	17 8	14 4
Average	27 3	16 6	115	38 5	14 0	15 4	9 5
			115	43 9	12 2	22 4	15 3
10 0	5 7		113		20 0		10 1
13 8	7 6		129		25 9		12 2
11 0	7 1		139		20 0		
14 3	6 2		132		20 0		
10 2	7 6		102		17 9		
10 5	6 7		139		12 9		
			84		16 1		
			121		16 1		
			115		16 1		
			120		8 5		
					12 2		
					16 2		
					11 6		
					17 9		
					17 9		
Average	11 6	6 8	158	36 6	16 1	17 9	9.7

For trypsin, in the first section (8 normal and 15 dehydrated samples) the estimations were carried out on 0.5 cc. of extract, incubated 1 hour; in the second section (6 normal and 6 dehydrated samples) the estimations were carried out on 2 cc. of extract, incubated  $\frac{1}{2}$  hour.

Estimations of trypsin were first made in duplicate with 0.5 cc. portions of the extract of pancreas; they were incubated for 1 hour. The average of these estimations shows that there were 16.6 units of trypsin per gm. of tissue in the fifteen dehydrated animals, as compared with 27.3 units in the eight normal animals.

Since the curve of tryptic digestion rises rather sharply at the outset and then flattens as the time increases, it was thought that a shorter incubation period would be preferable. A second series of estimations was carried out with 2 cc. of extract, incubated for  $\frac{1}{2}$  hour. Practically the same sort of result was obtained as in the first series of determinations. The dehydrated animals showed a definitely lower enzyme content than that shown by the normals; namely, an average of 6.8 as compared with 11.6 units (see Table I).

*Amylase*—Amylase is the carbohydrate-digesting enzyme of the pancreas. Its rôle in digestion consists in the breaking up of complex carbohydrates (polysaccharides) into simple diffusible sugars, such as maltose.

Forty-five estimations of amylase activity were carried out in duplicate on the same pancreatic extracts that were used in estimating tryptic activity.

The average amylase activity for the dehydrated animals (101 units per gm. of pancreas) is considerably less than that for the normal animals (158 units) (see Table I).

### *Intestinal Enzymes*

The entire small intestine was removed from each rat, opened longitudinally, gently washed with tap water, dried between filter papers, and then weighed and ground with sand, and a volume of 25 per cent glycerol equal to 10 times the weight was added. The tubes were left 18 hours at room temperature for extraction, and the erepsin and phosphatase activity of aliquot samples was determined.

*Erepsin*—It has been shown that the mucous membranes of the small intestine elaborate a specific enzyme which is concerned in the hydrolysis of those intermediate protein degradation products called peptides, which are formed by the action of the gastric pepsin and the pancreatic trypsin on the food proteins.

51 estimations of the ereptic activity of extracts of small intes-

tine were made in duplicate. The average of the figures for the dehydrated animals (16.1 units) is less than half the average value for the normal rats (36.6 units).

*Phosphatase*—Intestinal phosphatase, which is sometimes called nucleotidase, has to do with the hydrolysis of the organic compounds of phosphoric acid contained in the food. Such compounds include the phosphoric esters of sugars and related substances, the more complex nucleotides which have been studied extensively by Levene and Dmochowski (7), and the phosphorylated fats, lecithin and cephalin, the enzymic hydrolysis of which has recently been investigated by King (8).

Thirty-eight estimations of the phosphatase activity of the intestinal extracts were made in duplicate. The average of the results for the dehydrated rats (9.7 units) is found to be almost half that of the normal rats (17.9 units).

#### EXPERIMENTAL

*Trypsin*—Activation of the trypsin solution by enterokinase is necessary before its activity can be measured. The enterokinase solution is prepared by dissolving 2 gm. of dried intestinal mucosa in 100 cc. of 0.05 N  $\text{NH}_4\text{OH}$ . After the mixture has been kept for 2 hours at  $37.5^\circ$ , solution should be complete. As enterokinase is unstable in the presence of ammonia, however, it is necessary to rid the solution of ammonia by evacuation at the water pump for 1 hour. The solution should be stored in the ice box and made fresh each week.

For the determination of the tryptic activity the pancreas is extracted with dilute glycerol. A measured amount is activated for 30 minutes at  $37.5^\circ$  with 0.3 cc. of the enterokinase solution. Water is added to 5 cc., and 0.3 gm. of casein (5 cc. of 6 per cent solution at pH 8.9) is added, together with 2 cc. of N  $\text{NH}_2\text{-NH}_4\text{Cl}$  (1:2) buffer of pH 8.9. Four tubes are put up in this way; two of them are kept at  $37.5^\circ$  for exactly 60 minutes, and the other two are poured into 50 cc. of hot alcohol, which stops the enzyme action immediately. The tubes are washed out with an additional 50 cc. of hot alcohol, which has been made alkaline to thymolphthalein with a few drops of 0.2 N KOH. The mixture is titrated with 0.2 N KOH made up in alcohol. At the end of the 60 minute incubation period the other two tubes are treated in a similar manner.

The increase in the titratable acidity is measured by the difference between the potassium hydroxide used in the 0 and 60 minute tubes, and is taken as the measurement of the tryptic activity.

The unit of trypsin is defined as that amount of enzyme which will increase under these conditions the acidity of the mixture an amount equal to 1.05 cc. of 0.2 N KOH.

*Amylase*—Amylase activity is determined by estimating the amount of maltose liberated from starch by the pancreatic extract. The maltose formed is estimated by determining its reducing power against hypiodite by the Willstätter-Schudel method. In alkaline solution maltose reacts with iodine, with quantitative oxidation of the aldehyde group to carboxyl according to the equation,  $R \cdot CHO + I_2 + 3NaOH \rightarrow R \cdot COONa + 2NaI + 2H_2O$ . Excess iodine is added over and above that necessary to give complete oxidation of the maltose, and the residual iodine is determined by titration with thiosulfate.

In four cylinders are measured 25 cc. of freshly prepared 1 per cent starch solution; 10 cc. of phosphate buffer, pH 6.8; 1 cc. of 0.2 N NaCl; 0.5 cc. of enzyme solution; and 0.5 cc. of water. The enzyme action in two of the cylinders is stopped by the addition of 3 cc. of  $\frac{2}{3}$  N acid. The other two tubes are incubated for the stated period of  $\frac{1}{2}$  hour, and the enzyme action is stopped by the addition of the same amount of acid.

10 cc. portions are titrated with N NaOH to phenolphthalein to determine the amount of alkali necessary to neutralize the acid and the phosphate buffer. This amount is usually fairly constant from one determination to the next, and the titration may hence be usually omitted. 10 cc. portions are treated with the same amount of alkali in 50 cc. glass-stoppered flasks, and 2 cc. of 0.1 N  $I_2$  together with 3 cc. of 0.1 N NaOH are added. The flasks are kept for 15 minutes at 20°. 1 cc. of N acid is then added to liberate the remaining iodine, which is now titrated with 0.02 N thiosulfate, 3 drops of soluble starch solution being added for indicator. The difference between the thiosulfate titration in the 0 time and the  $\frac{1}{2}$  hour tests is taken as the measurement of the amylase activity, 1 cc. of 0.02 N thiosulfate being equal to 3.4 mg. of maltose.

The amylase unit is that amount of enzyme which will liberate 5 mg. of maltose under the above conditions.

*Example*—For six normal rats in which 0.5 cc. of a 1:20 extract was used, 22.4 mg. of maltose were formed. The 0.5 cc. contains therefore  $22.4/5$  units of amylase, and 1 gm. of tissue contains  $22.4/5 \times 20/0.5 = 179.2$  units.

*Erepsin*—Ereptic activity is determined by estimating the hydrolysis of the dipeptide glycylglycine by a measured amount of the enzyme solution. Erepsin acts on dipeptides to liberate free amino and carboxyl groups, the increase in either of which may be taken as a measurement of the enzyme activity. In the present method the carboxyl groups are measured by the Willstätter method, which takes advantage of the fact that the carboxyl group of the amino acid may be quantitatively titrated by alkali if the titration be carried out in alcohol. 5 cc. of 0.2 M (2.62 per cent) glycylglycine adjusted to pH 7.8 are added to 5 cc. of 0.1 M phosphate buffer of pH 7.8 and 0.5 cc. of intestinal extract. The volume is then made up to 15 cc. and the mixture is incubated for exactly 18 hours. It is then poured into 50 cc. of hot alcohol and the tube washed out with 50 cc. more of the hot alcohol (the alcohol should previously have been made just alkaline to thymolphthalein with KOH). Determinations are carried out in duplicate with 0 time controls.

The erepsin unit is that amount of enzyme which under the conditions of the experiment will liberate carboxyl groups equivalent to 1 cc. of 0.2 N KOH.

*Example*—0.5 cc. of 1:10 extract of rat intestine liberated carboxyl groups equivalent to 2.0 cc. of 0.2 N KOH. 1 gm. of tissue contains therefore  $2.0 \times 10/0.5 = 40$  units.

*Phosphatase*—The method used is that described by Kay (9). 5 cc. of Sørensen's glycine buffer (pH 8.9 at 37°), 5 cc. of 0.3 per cent sodium- $\beta$ -glycerophosphate, 0.5 cc. of the extract (adjusted to the same pH), and 2 drops of chloroform are added to each of four test-tubes. 2 cc. of 25 per cent trichloroacetic acid are added immediately to two of the tubes, and the other two are closed with well fitting rubber stoppers and incubated exactly 2 hours at 37.5° in a water thermostat, after which 2 cc. of the trichloroacetic acid are added to each. The mixtures are filtered through ash-free filter papers, and the inorganic phosphate estimated in 10 cc. of the filtrate by the colorimetric procedure described by King (1932) (10).



The phosphatase unit is defined as that amount of enzyme which will liberate 1 mg. of phosphorus under these conditions.

*Example*—0.448 mg. of phosphorus was liberated under the conditions of the experiment. The 0.5 cc. of intestinal extract represents, therefore, 0.448 unit of phosphatase. Since a 1:10 extract was used, then 1 gm. of tissue (equivalent to 10 cc. of extract) contains  $0.448 \times 10/0.5 = 8.96$  units.

#### DISCUSSION

An examination of the figures for the activity of the four enzymes (see Table I) shows that there is considerable individual variation between rats which had been similarly treated. This is particularly true in the case of amylase, in which the activity of the pancreatic extract from seventeen normal rats varies from 126 to 191 units, and the activity of extracts from twenty-eight dehydrated rats varies from 43 to 139 units. These variations are probably only to a minor extent due to errors in technique, as duplicate estimations gave an average experimental error of only 3 per cent.

The more likely explanation is that there was considerable variation in the amount of digestive activity in progress when the animals were killed. Some rats on autopsy were found to have stomachs greatly distended with food; others had a moderate amount of food in the stomach, while in others the stomach was practically empty. There also appeared to be more secretion and digestion products in the upper part of the small intestine in rats whose stomachs were distended with food, indicating a more active digestive process probably associated with an increased secretion of enzymes.

#### SUMMARY

Rats which have been subjected to prolonged dehydration by dry heat show a decrease in the production of both pancreatic and intestinal enzymes.

The diminished enzyme production in dehydrated animals is probably comparable to that found in dehydrated infants suffering from acute intestinal intoxication.

The authors wish to thank Dr. F. G. Banting and Dr. E. J. King for their constant interest and assistance throughout the course of the experiments and for defining the units of enzyme activity.

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## INORGANIC SALTS IN NUTRITION

### VII. CHANGE IN COMPOSITION OF BONE OF RATS ON A DIET POOR IN INORGANIC CONSTITUENTS\*

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A previous study (Brooke and Smith, 1933, *a*)<sup>1</sup> of the mineral metabolism of rats maintained on a ration extremely poor in inorganic salts disclosed the fact that, after a period of 90 days on the experimental diet, there existed in these animals a negative calcium and a positive phosphorus balance. The intake of calcium was negligible, whereas the phosphorus in the daily food consumed amounted to 11 to 15 mg., about half of which could be accounted for by the casein in the diet. Under the circumstances, it is difficult to believe that the calcium excreted arose primarily from the calcium phosphate portion of the bone salt. Considering the generally accepted view of the composition of the inorganic part of bone, it appears likely that carbonate was being withdrawn from the skeleton of these rats to a greater extent than was the phosphate. A demonstration of this process in the experimental animals in contrast with suitable controls would have a significant bearing upon the possibility of selective resorption of bone salts and upon the conception of the skeleton as a mineral reservoir. The present paper describes the changes in the composition of the bones of albino rats under conditions of strict limitation of inorganic salts in the diet.

#### EXPERIMENTAL

*Diets and Animals*—Three groups of nine animals each were used: Group I, normal age controls receiving *ad libitum*, a ration

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<sup>1</sup> Several corrections to the tables in this paper appear in the December, 1933, issue of the *Journal*.

adequate in all respects, Group II, calorie controls given a diet providing the same quantity of protein, vitamins, and salts as consumed by Group I but limited in energy intake to that ingested by Group III, on the low salt ration, and Group III, experimental animals fed in unrestricted quantity a ration poor in inorganic constituents.

The preparation, care, and behavior of the three groups have been described previously (Brooke and Smith, 1933, *a*). Table I presents the composition of the rations.

*Preparation of Bone Samples*—At the end of the experimental period (90 days) the animals were anesthetized with urethane

TABLE I  
*Composition of Experimental Diets*

Ingredients	Age controls	Calorie controls	Experi- mental animals
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Low ash casein.....	18	25.7	18
Dextrin.....	51	41.6	55
Hydrogenated fat (Crisco).....	27	27.0	27
Salts*.....	4	5.7	0
<div style="display: flex; align-items: center; justify-content: center;"> <div style="margin-right: 20px;">           200 mg. dried yeast            3 drops wheat germ oil            1 cc. alcoholic extract of wheat germ            5 drops cod liver oil         </div> <div style="font-size: 3em; margin-right: 10px;">}</div> <div>           daily adjuvants for            all groups         </div> </div>			

\* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 557 (1919).

administered by stomach tube. Both femurs were dissected out and treated in the following way. The bones were cleaned of all visible soft tissue, broken into two pieces, and dried to constant weight at 102°. Each pair of femurs was then wrapped in linen and tied with thread so that the bones could be cracked into small pieces without mechanical loss. Several such packages were defatted simultaneously in a large Soxhlet extractor. Four extractions, each of 1½ hours duration, proved adequate. A mixture of 8 parts of alcohol and 2 parts of ether which had been rendered just alkaline to phenolphthalein was employed for the first three times; in the final extraction ether alone was used and the residual ether was driven off at low temperature. The defatted femurs,

after removal of adhering marrow, were ground in an agate mortar to pass through a standard sieve with apertures 0.0137 inch square. Each pair of femurs, from an individual rat, constituted a separate sample.

*Analytical Procedures*—Calcium and phosphorus were determined on a solution prepared as described by Shear and Kramer (1928), except that small glass-stoppered weighing bottles were used instead of lead foil. About 50 mg. of the dried bone powder were transferred to 50 cc. flasks. The weight of the sample was determined to the nearest 0.05 mg. Solution was effected with hydrochloric acid and the protein precipitated with trichloroacetic acid (see Shear and Kramer, 1928; Bogert and Hastings, 1931). With No. 40 Whatman filter paper a clear filtrate was obtained.

Calcium was precipitated from 2 cc. of the filtrate as the oxalate, according to the procedure of Van Slyke and Sendroy (1929). The precipitate was washed according to the technique of Clark and Collip (1925) and finally titrated with 0.01 N permanganate from a 10 cc. burette graduated to 0.02 cc., with a platinum tip as advocated by Shohl (1928).

Phosphorus was determined by the method of von Lorenz (1901, 1907) which was adopted by Pregl in his system of micro-analysis. This procedure has given excellent results in our hands (Brooke and Smith, 1933, b). A series of determinations was run simultaneously; the phosphorus in each sample was estimated in duplicate and a satisfactory recovery was obtained *with every determination* within an error of  $\pm 1.0$  per cent.

The carbonate content of the bone was estimated on portions weighing about 30 mg. by means of the Van Slyke and Neill manometric gas apparatus. The procedure was essentially that of Shear and Kramer (1928). No difficulty was encountered in introducing the powdered bone into the reaction chamber. We preferred to use 40 per cent NaOH instead of KOH and found it necessary to raise the mercury two or three times before taking a reading on the manometer in order to obtain complete absorption of the carbon dioxide. The blank value was usually 3.0 mm.

#### DISCUSSION

The results are summarized in Table II. Inasmuch as the values from the individual animals are very uniform, only the

averages are presented. The data for calcium, phosphorus, and carbon dioxide are given both in percentages and as mm per 100 mg. of sample. The data for residual calcium were obtained by subtracting the figures in Line 6 from those in Line 2 and are expressed as mm. The ratios in Lines 9, 12, and 13 are calculated on the basis of mm rather than of percentage.

TABLE II  
*Analysis of Bone*

	Group I Age controls	Group II Calorie controls	Group III Low salt
1. Ca, per cent .....	30 41	29 43	17.56
2. " mm.....	0.760 $\pm$ 0.055*	0.736 $\pm$ 0.023*	0.439 $\pm$ 0.031*
3. P, per cent.....	13 22	13 44	8.49
4. " mm.....	0.427 $\pm$ 0.016	0.437 $\pm$ 0.020	0.274 $\pm$ 0.020
5. CO <sub>2</sub> , per cent. . . .	3 73	3.82	1.84
6. " mm.....	0.085 $\pm$ 0.005	0.087 $\pm$ 0.001	0.042 $\pm$ 0.003
7. $\frac{\text{Carbonate, mm}}{\text{Total Ca, mm}}, \%$	11.2	11.8	9.5
8. Residual Ca, mm .	0.676	0.649	0.397
9. $\frac{\text{Residual Ca, mm}}{\text{P, mm}} ..$	1.59	1.49	1.45
10. Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> from Ca, mm. ....	0.225	0.216	0.132
11. Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> from P, mm.....	0.213	0.218	0.137
12. $\frac{\text{Ca}_3(\text{PO}_4)_2, \text{mm}}{\text{CaCO}_3, \text{mm}}$ from (10).....	2.66	2.49	3.16
13. $\frac{\text{Ca}_3(\text{PO}_4)_2, \text{mm}}{\text{CaCO}_3, \text{mm}}$ from (11).....	2.52 $\pm$ 0.078	2.52 $\pm$ 0.147	3.28 $\pm$ 0.192

\* Standard deviation.

It is apparent that the concentrations of calcium, phosphorus, and carbon dioxide in the dry, fat-free bone of the animals maintained on the ration poor in salts are strikingly lower than those of either of the control groups, the percentage reduction of calcium being greater than that in phosphorus. This change in the unignited bone salt agrees with the previously observed distortion of the ratio of organic residue to actual ash in bones of animals

under essentially the same nutritional adjustment (see Smith and Schultz, 1930; Clarke, 1933).

Attention is directed to the ratio of residual calcium to phosphorus<sup>2</sup> (Line 9) in Table II. The close approximation to the theoretical value of 1.5 (on the molarity basis) for  $\text{Ca}_3(\text{PO}_4)_2$  in both control groups and even in the animals on the low salt ration is a strong indication that in the rat the calcium of the bone not bound as carbonate is present as the tertiary phosphate only. The data in Table II also have a bearing upon the chemical nature of the major portion of the inorganic part of bone. Morgulis (1931) in discussing the various suggested components of the complex, shows that strictly chemical methods of approach have not yielded unequivocal results. By means of comparisons of x-ray spectrograms of sections of bone and of known minerals, Taylor and Sheard (1929) and Roseberry, Hastings, and Morse (1931) have come to the conclusion that the inorganic elements in bone exist in a complex salt resembling the apatite series of minerals and expressed by the formula  $\text{CaCO}_3 \cdot n\text{Ca}_3(\text{PO}_4)_2$  where  $n$  has a value between 2 and 3. Bogert and Hastings (1931) have given this theory experimental support on the basis of analyses of normal ox bone. In the present investigation the data derived from the normal age controls and from the calorie controls (Lines 12 and 13) likewise support the foregoing view in so far as calcium, phosphorus, and carbonate are concerned and indicate that the value for  $n$  is about 2.5 for the albino rat of the age and under the experimental conditions herein employed.

The proportion of the total calcium of the bone as the carbonate (Line 6 divided by Line 2) is less in the group of animals given the low salt ration than in either of the control groups. All of the rats used in the present study were very close to 125 days old. Kramer and Shear (1928) observed that the percentage of total calcium as carbonate in the bones of normal rats 63 days old was

<sup>2</sup> The values for phosphorus in Table II have not been adjusted to account for the magnesium phosphate of the bone. Howland, Marriott, and Kramer (1926) have shown that if all the magnesium in bone is present as  $\text{Mg}_3(\text{PO}_4)_2$ , only 0.3 per cent phosphorus would be thus accounted for. Although this value is beyond the error of our analyses for phosphorus, the conclusions relative to the nature of the calcium phosphate in bone as stated herein are not altered by accounting for all the magnesium as the phosphate.



13 and at 150 days of age was 16, values somewhat higher than those of our normal group, 11.2 per cent, and of the calorie controls, 11.8 per cent. The average proportion of total calcium as the carbonate in the experimental rats was 9.5. A further indication of the decrease in carbonate in the bones of the group on the low salt ration is the marked increase in  $\text{Ca}_3(\text{PO}_4)_2$  to  $\text{CaCO}_3$  ratio (Lines 12 and 13, Table II). This is very pronounced whether the tricalcium phosphate concentration is calculated from the calcium or from the phosphorus values. When conventional statistical methods are applied to the data in Line 13, Table II, it is apparent that the difference between the average ratio of the group on the low salt ration and that of the age controls is highly significant ( $D/PEd = 16.2$ ). The same conclusion is warranted when the comparison is made with the calorie controls ( $D/PEd = 14.6$ ). From the foregoing discussion it is plain that the calcium being lost from the body of the animals on the salt-poor ration (see Brooke and Smith, 1933, *a*) is provided to a greater extent from the carbonate of the bone salt than from the phosphate.

A shift in the phosphate to carbonate ratio is not unknown in normal animals. Morgulis (1931) has shown that a variation in this value exists between various species; Kramer and Shear (1928) and Neal, Palmer, Eckles, and Gullickson (1931) have demonstrated that this ratio decreases with advancing age both in rats and in cattle. The value of the ratio has been altered experimentally. Thus, Neal, Palmer, Eckles, and Gullickson (1931) brought about a lowered ratio in cattle by feeding rations poor in phosphorus and a slight decrease on diets low in calcium. The proportion of phosphate to carbonate is markedly diminished in rachitic rats and infants (Howland, Marriott, and Kramer, 1926) and inanition leads to a decrease in the proportion of carbonate (Gusmitta, 1893). The bones of pigs consuming a ration with 0.029 to 0.097 per cent fluorine contained normal percentages of calcium and phosphorus but a decreased percentage of carbon dioxide (Kick, Bethke, and Edgington, 1933). That the calcium carbonate in bone salt is promptly effective in neutralizing ingested acid has been demonstrated by Irving and Chute (1932-33) in experiments with rats and guinea pigs. In the present study the composition of the bone salt of animals on the mineral-deficient diet showed a change which cannot be correlated with age or

with partial inanition but which occurs as a result of the demand for calcium under the severely restricted nutritive conditions imposed.

In the experimental animals on the low salt ration in the present investigation, the pathological picture of extreme osteoporosis (Swanson, 1930) and the uniformly negative calcium balance throughout the experimental period (90 days) point to a withdrawal of salts from the skeleton rather than to an abnormal deposition. It appears, then, that there is demonstrated in the group of experimental animals in the present study a differential resorption of the components of the bone salt. This is contrary to the principle enunciated by Armsby (1917), namely that a draft upon the skeleton for a single element "could be met only by the mobilization of an amount of total bone ash containing the requisite quantity" of the element in question, and the present observation indicates again that the mineral reservoir in the bones can vary in qualitative make-up as well as in quantity.

#### SUMMARY AND CONCLUSION

In rats restricted for 90 days to a ration extremely poor in inorganic salts, there occurs a reduction in the calcium, phosphorus, and carbonate of the unignited bones. The  $\text{Ca}_3(\text{PO}_4)_2$  to  $\text{CaCO}_3$  ratio in the bone salt of these animals is significantly increased over that of the control animals, but the ratio of non-carbonate calcium to phosphorus remains normal. Mild inanition alters neither the phosphate to carbonate ratio nor the residual calcium to phosphorus ratio. These chemical changes are interpreted to mean that the persistent negative calcium balance in the rats on the low salt ration exists at the expense of the carbonate to a greater extent than at the expense of the phosphate.

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## A METHOD FOR THE ANALYSIS OF TISSUES\*

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The object of tissue analysis is to secure an accurate representation of the condition of the tissue in its normal state. This can be accomplished only by first maintaining conditions which prevent further changes during the final preparation of the tissue. In the procedures now in general use the tissue is rapidly frozen in liquid air (1) or a CO<sub>2</sub> snow-ethyl chloride mixture. It is then either thinly sliced while still frozen and immersed in trichloroacetic acid (2) or ground in a cooled mortar containing sand and various precipitants (1, 3). We have found these methods to be not only tedious but clumsy, particularly when multiple samples are being prepared. Furthermore, losses from spattering and evaporation may occur in the use of the open mortar for the grinding of the tissue. In the procedure to be described, the authors have attempted to simplify the technique in order to minimize the possibility of errors in the sampling and preparation of the tissue and to facilitate the handling of multiple specimens. Tissue samples of from 1 to 10 gm. may be treated with equal facility.

### *Apparatus*

The apparatus necessary for this procedure is shown in Fig. 1. The tissue crusher, which is shown in Fig. 2, consists of a base, *A*, attached to a round solid piece of steel, *B*, which serves as the anvil upon which the tissue is crushed. A steel cylinder, *C*, is permanently fastened to the anvil as shown. A second removable cylinder, *D*, rests upon the anvil and fits snugly around the raised central portion. Small holes bored in cylinder *D* permit the rapid

\* This study was aided by grants from the Bartlett Memorial Fund and the Douglas Smith Foundation for Medical Research, University of Chicago.

escape of air as the plunger, *E*, descends. The entire apparatus is surrounded by the removable cylinder, *F*. A U-shaped trough fastened to cylinder *C* traverses the space between cylinders *C* and *F*. The tissue, after being crushed, can thus be conveniently

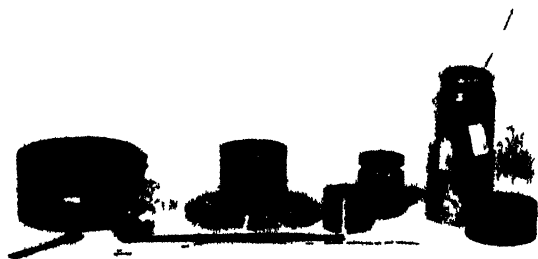


FIG. 1 Auxiliary apparatus and unassembled parts of tissue crusher

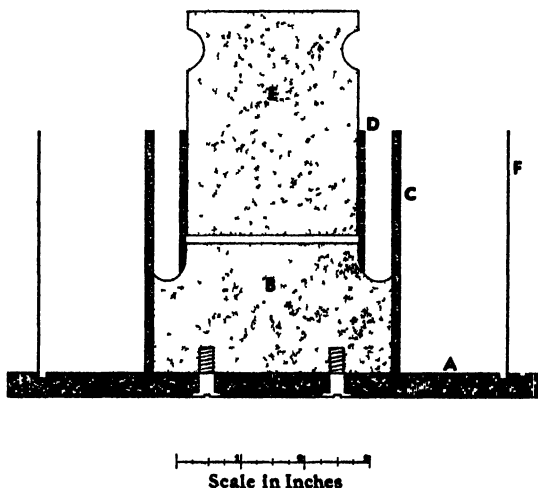


FIG. 2. Tissue-crushing apparatus

swept from the moat of the anvil into the flask containing acid and precipitating agents.

In preparation for an experiment the crusher is assembled with the plunger in place and the entire apparatus filled with CO<sub>2</sub> snow. 5 pounds of compressed CO<sub>2</sub> snow suffice to keep the ap-

paratus cool for at least 2 hours. About 10 minutes are required for complete cooling. Another receptacle containing CO<sub>2</sub> snow is kept available for cooling the plunger when this is removed from the apparatus. After the apparatus is cooled, CO<sub>2</sub> snow and condensed moisture remaining on the anvil and in the spout are removed by means of a brush or a dry towel.

### *Procedure*

Because the tissues can be prepared for analysis so quickly we usually obtain multiple samples from the animal or animals as the experiment may demand, and store them in the Dewar flask con-

TABLE I

#### *Effect of Liquid Air on Moisture Content of Lung Tissue*

Before obtaining the samples the animal was exsanguinated. 2.5 to 4.0 gm. of crushed tissue were dried 15 hours at 50-60° and 5 hours at 105°.

Length of time in liquid air	Moisture content
	<i>per cent</i>
3 min.	78.9
	79.0
	79.1
1 hr.	79.8
	79.9
	80.1
3.25 hrs.	79.0
	79.1
	79.1

taining the liquid air until ready for further preparation. An S-shaped pin, attached to a label by a short length of thread, is hooked into each sample just before plunging into the liquid air (see Fig. 1). No change in moisture content of the samples has been noted after remaining in the liquid air for as long as 3 hours, as is shown in Table I.

The tissue sample (1 to 10 gm. in weight) which has thus been frozen in the liquid air is placed within the inner cylinder of the cooled tissue crusher. The plunger is inserted and immediately given several heavy blows with a sledge hammer. The plunger is then removed and the compressed mass of crushed tissue stirred and recrushed. This is repeated one or more times depending on

the type of tissue being treated. The tougher tissues, such as skin, require three to four repeated crushings. When this part of the process is completed, the plunger and cylinder *D* are removed and the compressed cake of frozen pulverized tissue broken into samples of suitable size.

One or more aliquots of the pulverized tissue cake are then brushed through the trough into a stoppered tared flask or flasks which contain accurately measured volumes of precipitant and acid. The flask is immediately stoppered and shaken vigorously. The shaking is repeated at short intervals to facilitate the breaking up of the particles of tissue and rapid penetration of the acid. The flask and its contents are subsequently weighed after which the precipitation of the proteins is completed.

After sampling, the remaining traces of tissue are removed by the brush and dry towel. Cylinder *D*, which has been kept cool in the meantime in the receptacle containing CO<sub>2</sub> snow, is then replaced and the apparatus is ready for the next sample. The entire operation of crushing and sampling can be carried out in 1 to 2 minutes.

#### *Analysis for Glucose and Lactic Acid*

The proteins are precipitated by Zn(OH)<sub>2</sub> (Somogyi (4)). Two solutions are required. Solution 1, 20.0 gm. of ZnSO<sub>4</sub>·7H<sub>2</sub>O, 50 cc. of 10 N H<sub>2</sub>SO<sub>4</sub>, and 20.0 cc. of 1.00 per cent glucose are diluted to exactly 1 liter. Solution 2, NaOH is adjusted exactly to neutralize an equal volume of Solution 1, with a reaction distinctly pink to phenolphthalein. 5 cc. of each of these solutions are used for every gm. of sample.

Because of the large volume occupied by the protein precipitate, only about one-half of the filtrate with the usual 10-fold dilution is available for analysis. Thus, a 2 gm. sample would yield available filtrate representing about 1 gm. To increase the yield of filtrate we recommend a 15-fold dilution. Tissues often may contain less glucose than does the blood. Sugar analyses in such tissues, diluted 15-fold, are not very accurate, since the copper reduction is small. The accuracy is increased (error  $\pm 2$  mg. per cent) by the addition of glucose, which brings the titrations into a more accurate range (above 100 mg. per cent).

10 cc. of Solution 1 (for a sample estimated to weigh 2 to 3 gm.)

are accurately measured and transferred to a wide mouth flask which is then tightly (rubber) stoppered and weighed. The sample having been introduced and mixed with Reagent 1, the flask and contents are weighed again. The necessary additional volume of Solution 1 is then added from a burette. An equal volume of distilled water, minus the weight of the sample, is added from a burette. Finally, an exactly equal volume of Solution 2 is added.

TABLE II

*Analysis of Skin and Lung Tissue of Dogs*

Samples of skin and blood of Dogs 1 and 2 were taken while under amytal anesthesia. Dogs 3, 4, and 5 had experimental lobar pneumonia; they were electrocuted (110 volt alternating current for 5 seconds). Samples were taken within 45 seconds after death. The results shown represent only the uninfected lobes of the lung. 3 to 8 gm. samples were analyzed.

Dog No.	Sample	Glucose	Lactic acid
		mg. per cent	mg. per cent
1	Skin, left fore leg	74	17.5
	" right " "	70	17.8
	" left hind leg	64	14.7
	" right " "	70	15.6
	Blood	88	15.5
2	" (Folin-Wu precipitation)	97	15.4
	Skin, right hind leg	58	7.2
	" left " "	60	12.6
	Blood, beginning of experiment	87	8.9
	" end " "	90	9.0
3	Lung	66	18
	Blood from heart after death	72	17
4	Lung	70	25
	Blood from heart after death	78	26
5	Lung	62	32
	Blood from heart after death	64	21

The flask is immediately stoppered and shaken. At least 30 minutes are allowed for equilibrium to take place, during which time the flask is frequently shaken. The contents are finally filtered through a dry fluted filter paper.

Determinations for glucose are made in duplicate by the Shaffer-Hartmann (5) method. An aliquot of the remaining filtrate is treated with  $\text{CuSO}_4$  and  $\text{Ca(OH)}_2$  and analyzed in duplicate for lactic acid (6).



A blank determination for both lactic acid and glucose is also made with equal volumes of H<sub>2</sub>O and Solutions 1 and 2.

#### DISCUSSION

In any analytical procedure of this type one is confronted with the problem of considering the amount of postmortem change which may have occurred in the tissue prior to its final preparation for analysis. The extent of these changes is difficult to evaluate, particularly in warm blooded animals, for generally accepted criteria for judging the extent of these changes are lacking. Normal tissues are presumably in equilibrium with blood so that analysis of the blood probably offers the best means of estimating these changes. Since tissues, on removal from contact with the circulating blood, rapidly increase their content of glucose and lactic acid, it would be presumed that minimum change would be indicated by values for these metabolic products equal to, or less than, that of the blood sample obtained simultaneously with the removal of the tissue. A comparison, then, between the glucose and lactic acid content of the tissue and the blood should offer reasonable criteria for estimating the degree of postmortem change.

Illustrative data for the glucose and lactic acid content of skin and lung obtained by the procedure described, with control values for blood, are given in Table II. In Dogs 1 and 2 the lactic acid content of skin agrees closely with that of the blood. The glucose values are from 15 to 30 mg. per cent lower in the tissues. A similar relationship obtains in the data on lung tissue (Dogs 3 and 4), although the difference between the glucose values of blood and lung tissue are not as great as in the skin. This may be due to the higher vascularity of the lung tissue. In Dog 5, however, the lactic acid content of the lung tissue is far larger than that of the blood, while the glucose content is almost equal to that of the blood. These latter results, we believe, suggest postmortem changes which have occurred in the tissue during the process of its preparation for analysis.<sup>1</sup>

We wish to thank Mr. Theodore Brook for technical assistance in this work.

<sup>1</sup> This point will be discussed at greater length and more complete data will be published in subsequent papers from this laboratory.

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# THE IRON CONTENT OF THE WHOLE BLOOD OF NORMAL INDIVIDUALS

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During the course of an investigation involving the determination of blood iron values in normal subjects, it became evident that the results were consistently higher than the figures recently reported for human blood by Murphy, Lynch, and Howard (1) and Reich and Tiedemann (2). Furthermore, hemoglobin values calculated on the basis of their iron determinations differed considerably from values obtained by them, with the Sahli and Newcomer methods. Oxygen capacity figures calculated from their iron determinations were also lower than those generally considered normal in the literature. Since the oxygen capacity method is the standard method of determining the hemoglobin content of blood, simultaneous determinations of oxygen capacity by the Van Slyke and Neill method (3) and iron determinations by the Kennedy method (4) were made on the blood of a series of normal men and women. Since the completion of this work, Sachs, Levine, and Appelsis (5), using the Wong method (6), have reported iron values for the blood of normal men that are in accord with the data recorded in this paper, although their figures for normal women are definitely lower.

In order to determine whether the discrepancies in the iron values quoted in the papers above might be explained by the methods used for estimating the iron content, a comparison was also made between the Kennedy and the Wong methods.

## *Methods*

The subjects used for these experiments—doctors, nurses, students, and technicians—were all apparently normal individuals

between the ages of 20 and 40 years. Samples were obtained for analysis by withdrawing approximately 10 cc. of venous blood from the large arm vein of each individual and transferring the blood to a bottle containing iron-free sodium oxalate. All collections were made between the hours of 8.30 a.m. and 10 a.m. during the months of July and August.

TABLE I

*Results of Analyses for Iron Content and Oxygen Capacity on Blood of Ten Normal Men and Ten Normal Women with Oxygen Capacity Also Calculated from Total Iron Content and Protein Iron Content*

Case No.	Sex	Red blood cells	Iron	O <sub>2</sub> capacity			Sex	Red blood cells	Iron	O <sub>2</sub> capacity		
				Calculated from total Fe	Calculated from protein Fe	Experimental				Calculated from total Fe	Calculated from protein Fe	Experimental
		mil- lions per c.mm.	mg. per 100 cc.	vol. per cent	vol. per cent	vol. per cent				vol. per cent	vol. per cent	vol. per cent
1	M.	6.03	57.1	22.8	22.4	22.6	F.	5.03	43.9	17.6	17.2	16.5
2	"	5.91	53.5	21.4	21.0	21.0	"	5.23	45.9	18.4	18.0	18.5
3	"	5.69	51.3	20.5	20.1	20.4	"	4.70	42.0	16.8	16.4	17.4
4	"	5.51	50.5	20.2	19.8	19.4	"	4.85	45.5	18.2	17.8	19.0
5	"	5.22	49.8	19.9	19.5	20.1	"	4.32	48.2	19.3	18.9	18.4
6	"	5.45	48.1	19.2	18.8	19.4	"	4.81	44.0	17.6	17.2	17.1
7	"	5.66	50.5	20.2	19.8	20.7	"	5.12	46.5	18.6	18.2	18.7
8	"	5.61	53.0	21.2	20.8	20.9	"	5.46	49.8	19.9	19.9	19.8
9	"	5.50	52.3	20.9	20.5	20.5	"	5.29	45.7	18.3	17.9	16.7
10	"	5.24	49.3	19.7	19.3	20.1	"	5.27	46.5	18.6	18.2	18.4
Average..		5.58	51.5	20.6	20.3	20.5		5.01	45.8	18.3	18.0	17.7

The oxygen capacity was then determined by the method of Van Slyke and Neill (3). Iron was determined by the Kennedy method (4). The blood was digested without difficulty in 25 × 200 mm. Pyrex glass test-tubes instead of the Kjeldahl flasks recommended by Kennedy.

In comparing the Kennedy and Wong methods, the same pipette was used to measure both blood samples, 1 cc. samples being used for both methods. We found better results could be obtained in the Wong method when 4 cc. of distilled water were added to the blood before the concentrated sulfuric acid was added.

The red blood cell counts were made on the samples obtained from the arm vein. Standard pipettes and counting chambers were used.

### Results

The results of the analyses are shown in Table I. The iron values are converted into oxygen capacity figures by multiplying

TABLE II  
*Results of Determination of Iron in Whole Blood of Normal Individuals by Kennedy and Wong Methods*

Case No	Fe per 100 cc blood		Red blood cells  millions per c mm.
	Kennedy method  mg	Wong method  mg	
1	51 5	50 5	5 05
2	52 5	47 6	5 30
3	55 3	52 7	5 47
4	53 8	50 5	4 80
5	50 8	47 2	4 79
6	51 0	49 3	4 85
7	54 5	53 2	5 16
8	55 3	54 4	5 77
9	53 8	49 5	5 31
10	57 2	55 6	5 57
11	51 6	45 4	6 16
12	52 1	50 3	5 08
13	51 3	49 8	4 67
14	52 7	48 8	5 48
15	56 2	52 1	5 80
Average	53 31 $\pm$ 1 21* $\pm$ 1 94†	50 26 $\pm$ 1 83* $\pm$ 2 72†	5 284

\* Probable error.

† Standard deviation.

the mg. per cent of iron by 0.400. (Since 1 mole of oxygen occupies 22,400 cc. at 0°, 760 mm., the molal ratio Fe:O<sub>2</sub> = 1:1 corresponds to a ratio of gm. of Fe to cc. of O<sub>2</sub> = 56:22,400 = 1:400, or mg. of Fe to cc. of O<sub>2</sub> = 1:0.400.) McIntosh (7) has shown that normal blood contains 1.02 mg. of non-protein iron per 100 cc. Therefore we have also converted the iron values to oxygen capacity after subtracting this figure from the total iron values.

In Table II are shown the mg. of iron in 100 cc. of the same blood analyzed by the Kennedy and Wong methods.

#### DISCUSSION

The data presented in this paper show that there is a close agreement between the hemoglobin content of normal human blood as determined by the oxygen capacity method and the Kennedy iron method. Therefore, the determination of iron offers an easy means of estimating the hemoglobin content of blood or of standardizing colorimetric methods of estimating hemoglobin. If we add the figures of eight of the cases from Table II, which were not included in Table I, the average iron content of the blood of eighteen normal men, determined by the Kennedy method, varied from 49.3 to 57.2 mg. per 100 cc., with an average of 52.5 mg. per 100 cc. of blood. For the ten normal women the iron content varied from 42.0 to 49.8 mg. per 100 cc. of blood, with an average of 45.8 mg.

In Table III the results of the recent iron determinations in human blood and the oxygen capacity and hemoglobin figures calculated from the iron content are compared to the hemoglobin figures recorded in the literature for normal men and women. The data of Reich and Tiedemann are not included in Table III because their normals can hardly be called that in the strict sense of the word. The iron values reported in this paper agree with the values of hemoglobin reported on larger series of cases by Haden (8), Osgood (9), and Wintrobe and Miller (10). The results of Murphy and coworkers are definitely lower than would be expected for blood with normal hemoglobin content.

As shown in Table II, the Kennedy method gave distinctly higher results than the Wong method. Although the simplicity of the Wong method recommends its use, in our experience the Kennedy method proved to be more satisfactory.

#### SUMMARY

1. There is a close agreement between the hemoglobin content of blood as determined by its iron content and oxygen capacity.
2. The blood iron content of eighteen normal men, determined by the Kennedy method, varied from 49.3 to 57.2 mg. per 100 cc., with an average of 52.5 mg.

TABLE III

*Results of Recent Iron Determinations in Human Blood, and Oxygen Capacity and Hemoglobin Values, Calculated from Iron Content, and Normal Values of Hemoglobin and Oxygen Capacity As Recorded in Recent Literature*

Authors	Red blood cells	Men				Women			
		Iron	O <sub>2</sub> capacity	Hb		Red blood cells	Iron	O <sub>2</sub> capacity	Hb
	mil- lions per c.mm.	mg. per 100 cc.	vol. per cent	gm. per 100 cc		mil- lions per c mm.	mg. per 100 cc.	vol. per cent	gm. per 100 cc.
Helmer and Emerson.....	5 51	52.50	21 0	15 66		5 01	45.75	18 3	13 68
Sachs <i>et al.</i> (5).....	5 00	50 01	20 0	14 93		4 46	42 67	17 0	12 74
Murphy <i>et al.</i> (1)....	5 35	44 84	17 9	13 38		4 92	42.48	17 0	12 68
Sackett (11).....	5 09	56 02	22 4	16 71		4 68	51 20	20 5	15 28
Haden (8).....	5 08		21 2	15 83		4 26		17 3	13 34
Osgood (9) . . . .	5 40			15 80		4 80			13 70
Wintrobe and Miller (10) .	5 85			15 87					

3. The blood iron content of ten normal women varied from 42.0 to 49.8 mg. per 100 cc., with an average of 45.8 mg.

4. Higher iron values were obtained with the Kennedy method than with the Wong method.

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# THE MUCILAGE FROM SLIPPERY ELM<sup>1</sup> BARK

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The bark of slippery elm, *Ulmus fulva*, contains large amounts of mucilage together with starch and calcium oxalate (1). Schirmer (2) reported that the mucilage obtained from this bark contained 1.4 per cent nitrogen, 12.18 per cent pentosan, 10.26 per cent methylpentosan, and 13.72 per cent fermentable sugars calculated as glucose. He reported glucose, fructose, and galactose among the sugars.

The mucilage is a mixture of polyuronides which is contaminated by varying amounts of calcium oxalate. This latter is not a part of the mucilage proper but is merely dissolved out of the bark along with the mucilage. The mixture of polyuronides is difficult to separate into pure compounds. However, after it had been partly hydrolyzed the following substances were found present: (a) *l*-rhamnose galacturonide; (b) a disaccharose uronide; (c) the sugars *d*-galactose and *l*-rhamnose; (d) a colored non-saccharine material which will be called an *X* body; and (e) calcium oxalate. Indications point to the presence of three other substances, namely a pentose sugar, a methylated aldose sugar, and a methylated uronic acid. Contrary to the statement of Schirmer (2) neither glucose nor fructose was found.

## EXPERIMENTAL

**Bark Used**—The material used in the preparation of the mucilage was the inner bark of slippery elm.<sup>1</sup> This bark gave a strong qualitative test for starch and for calcium oxalate. Analysis showed it to contain 7.63 per cent moisture, 7.70 per cent ash, 5.9 per cent pentosan after correcting for the furfural formed from the

<sup>1</sup> The bark was purchased from a chemical supply house.

uronic acid ((3) p. 71), 4.72 per cent methylpentosan, and 12.68 per cent uronic acid anhydride ((3) p. 71).

*Preparation of Mucilage (2, 4)*—500 gm. of bark were cut into shavings and mixed with 10 liters of water. After 24 hours the mucilaginous solution was pressed through cloth and mixed with twice its volume of 95 per cent ethanol. The liquid was siphoned from the flocculated mucilage. The latter was again mixed with ethanol and allowed to stand for an hour. It was then filtered off, washed with ethanol and ether, and dried. The shavings were extracted twice more, each time with 6 liters of water for 24 hours

TABLE I  
*Analysis of Crops 1, 2, and 3 of Slippery Elm Mucilage and of Purified Mucilage*

	Crop 1	Crop 2	Crop 3	Purified mucilage*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Ash.....	8.70	10.55	12.30	1.00
Calcium oxalate.....	6.25	13.00	18.60	0.00
Carbon dioxide.....	6.50	5.80	5.26	8.25
Uronic acid.....	28.65	25.57	23.10	36.40
<i>l</i> -Rhamnosan.....	13.60	12.00	11.00	13.70
Pentosan.....	3.60	3.50	2.60	6.50
Galactan.....	9.60	10.80	9.00	11.00
Methoxyl.....	3.51			4.60
X body.....	4.60	5.10	6.30	6.00

\* The equivalent weight from the CO<sub>2</sub> determination was 533.

and the mucilage isolated as described. The three crops of mucilage weighed respectively 45 gm., 25 gm., and 12 gm., or a total of 16.4 per cent of the weight of bark used. Considerable mucilage remains in the bark after the third extraction.

In order to purify the mucilage 25 gm. were mixed with 4 liters of water and allowed to stand overnight. The solution was mixed with 1 liter of 18 per cent hydrochloric acid. This solution was repeatedly pressed through cloth. The purified mucilage, isolated as already described, was thoroughly washed with ethanol. By this procedure all of the calcium oxalate and a large part of the metallic ions are removed. The resulting product consists chiefly of the free mucilage acid. Its physical properties, however, are

not appreciably different from the original mucilage. The results obtained on analysis of the mucilage are given in Table I.

*Methods of Analysis*—The methods used in the determination of ash, uronic acid, methoxyl, pentosan, and methylpentosan are standard and need no description. The galactan was determined by oxidation to mucic acid of a portion of the sugar resulting from hydrolysis of the mucilage. Before this oxidation all uronic acid was removed as the calcium salt (5). The X body was determined by hydrolyzing the mucilage in hydrochloric acid and filtering off the insoluble X body. The oxalic acid was precipitated, as calcium oxalate, out of the filtrate from the determination of the X body.

*Partial Hydrolysis of Mucilage*—The mucilage was separated by hydrolysis into three portions; namely, the insoluble X body, the salts of sugar-uronic acid complexes, and a syrup containing the sugars. In one case 24 gm. of mucilage, containing 12 per cent moisture and 6 per cent calcium oxalate and corresponding to 19.7 gm. of anhydrous, oxalate-free material, were mixed with 1 liter of 4 per cent sulfuric acid solution and heated for 18 hours in a bath of boiling water. The flocculent, insoluble, slightly pink-colored X body was filtered off and found to weigh 1 gm. The filtrate from the X body was neutralized with barium carbonate and barium hydroxide. Eventually 10.4 gm. of the barium salt of an aldobionic acid were precipitated with alcohol and thus separated from the sugars which had been liberated from the mucilage by hydrolysis and are soluble in alcohol. The alcohol solution of the sugars was concentrated and the gum sugar found to weigh 8 gm. The percentage yield of products from the 19.7 gm. of mucilage were: X body 5 per cent, barium salts 50.8 per cent, gum sugar 40.6 per cent.

### *Constituents of Mucilage*

*Uronic Acid*—The naphthoresorcinol test ((3) pp. 55–57, (6)) and the yield of carbon dioxide by the method of Lefèvre (7) established the presence of one or more uronic acids. Galacturonic acid was identified as one of these acids by the method of Heidelberg and Goebel (8). In one case 3 gm. of barium salt gave 0.8 gm. of mucic acid which melted at 217°. While the method is

not quantitative, the indications are that the major portion of the uronic acid is present as galacturonic acid.

*l-Rhamnose*—This sugar was identified as a constituent of the mucilage by following the procedure described by Anderson and Crowder (9). The crystalline sugar melted at 94–96°. 2 minutes after solution it showed a slight levorotation. The final  $[\alpha]_D^{25} = +9.4^\circ$ . These are the properties of *l*-rhamnose. This sugar was also isolated from the syrup obtained by direct hydrolysis of the mucilage.

*d-Galactose*—8 gm. of gum sugar obtained by hydrolysis of 19.7 gm. of mucilage were dissolved in a small volume of glacial acetic acid, seeded with *d*-galactose, and allowed to stand in the refrigerator for 2 weeks. The crystalline sugar weighed 2 gm. It was identified as *d*-galactose by its  $[\alpha]_D^{25} = +80^\circ$  and by quantitative conversion to mucic acid which melted at 217°.

*X Body*—The purified mucilage is a white powder. When it is mixed with 4 per cent hydrochloric acid it gradually forms a colorless viscous solution. When this solution is heated in a bath of boiling water a slightly pink, flocculent precipitate gradually forms. This is the *X* body. After the solution has been heated for 3 hours the *X* body is filtered off and found to weigh approximately 6 per cent of the weight of the mucilage used.

*Methoxyl Group*—The presence of an ether-linked methoxyl group in the mucilage was established by the method of von Fellenberg (10) and Denigès (11). The approximate position of the methoxyl group in the mucilage molecule was determined by examination of the various products that result from hydrolysis of the mucilage. Neither the *X* body nor the less soluble portion of the salts gave any test for a methoxyl group. On the other hand the sugar syrups, after removal of *X* body and salts by solution in a mixture of absolute alcohol and ether, gave a strong test for an ether-linked methoxyl group. This indicates that the methoxyl is connected with the sugar portion of the molecule. The very soluble portion of the salts of the uronic acid-sugar complex also gave a test for the methoxyl group.

In order to obtain more evidence as to the position of the methoxyl group, a study was made of various non-crystalline sugar syrups obtained by hydrolysis of the mucilage. When the total sugar in one such syrup was determined by Fehling's solu-

tion ((3) p. 121) and the results calculated as *d*-galactose, approximately 90 per cent of the syrup was found to be sugar. The method of Cajori (12) showed that 90 per cent of the syrup was an aldose sugar. Pentosan determination indicated that the syrup consisted of a mixture of pentose and hexose sugars. This mixture gave 6.74 per cent methoxyl and a negative naphthoresorcinol test for a uronic acid. The aldose sugars in the mixture were oxidized to the corresponding monobasic acids by a water solution of bromine. The calcium salts of this mixture of acids, after purification, gave 13.44 per cent calcium oxide and 6.43 per cent methoxyl. This would indicate that between one-third and one-half of the syrup was present as a methylated aldose sugar. It thus appears that in some cases the methoxyl group is attached by an ether linkage to the sugar portion of the mucilage and possibly also in some cases to the uronic acid portion of the mucilage.

*Sugar-Uronic Acid Complexes*—When the mucilage is hydrolyzed a mixture of sugar-uronic acid complexes, or sugar uronides, results. Among the components of this mixture are the salts of: (a) the free uronic acid, (b) the uronic acid combined with 1 molecule of sugar with one free aldehyde group, (c) the uronic acid combined with 2 molecules of sugar with one free aldehyde group. By dissolving this mixture in water and adding successively larger volumes of ethanol it can be separated into fractions which approximate the composition of definite compounds. This method was used in separating the components of the following mixture of salts.

140 gm. of calcium salts obtained by hydrolysis of 285 gm. of mucilage were dissolved in 285 cc. of water and 285 cc. of ethanol were added. This precipitated 63 gm. of calcium salt, Fraction A. To the filtrate from Fraction A were added 285 cc. of ethanol. This precipitated 23 gm. of calcium salt, Fraction B. To the filtrate from Fraction B were added 560 cc. of ethanol. This precipitated 12 gm. of calcium salt, Fraction C. The filtrate from Fraction C was concentrated to a gum. This was dissolved in water and decolorized by carbon and again concentrated to a syrup. A large volume of ethanol was added to this syrup and 35 gm. of calcium salt, Fraction E, were obtained. Analysis showed that calcium salts, Fractions B and C, approximated in composition the calcium salt of *l*-rhamnose galacturonide, while

Fraction E approximated in composition the calcium salt of a disaccharose uronide. Fractions B and C and Fraction E were accordingly purified further by solution in water and precipitation by ethanol and examined as described below.

*l-Rhamnose Galacturonide*—Calcium salt, Fractions B and C, gave on analysis the following results: carbon dioxide found 12.10 per cent, theory 12.26 per cent; calcium oxide found 9.00 per cent, theory 7.80 per cent; aldehyde group, CHO, found 8.55 per cent, theory 8.08 per cent; equivalent weight calculated from the carbon dioxide determination 364, theory 359;  $[\alpha]_D^{25} = +65.6^\circ$ . In this case the results approximate closely the theory. This salt has already been shown to contain *l*-rhamnose combined with galacturonic acid. It gave no test for a methoxyl group.

In order to determine the structure of this aldobionic acid it was oxidized to the dibasic acid by the method of Goebel (13). The oxidation was allowed to proceed for several days instead of the brief period allowed by Goebel. After oxidation the salt still gave a strong naphthoresorcinol test for a uronic acid and evolved carbon dioxide when heated with hydrochloric acid. It gave no test for a methoxyl group. On analysis the oxidized salt gave the following results: carbon dioxide found 11.00 per cent, theory 11.17 per cent; calcium oxide found 14.11 per cent, theory 14.21 per cent; equivalent weight from the carbon dioxide determination 400, theory 394;  $[\alpha]_D^{25} = +71.5^\circ$ . These results prove conclusively that the glucosidic union involves the aldehyde group of the uronic acid. The structure of the aldobionic acid is thus similar to that suggested by Anderson and Crowder (9) for an aldobionic acid obtained from flaxseed mucilage.

*Disaccharose Uronide*—A portion of calcium salt, Fraction E, prepared as already described, was oxidized to a dibasic acid by the method of Goebel (13). The results obtained on analysis of the salt before and after oxidation are given in Table II. Both the oxidized and the unoxidized salts gave strong naphthoresorcinol tests for a uronic acid as well as a strong positive test for an ether-linked methoxyl group.

The data in Table II show that Fraction E approximates closely the composition of a disaccharose uronide. It apparently consists of a uronic acid, a pentose or methylpentose, and a hexose or methylated hexose, with one aldehyde group free. In order to

determine the relative position of the hexose in the unoxidized salt, a portion of it was hydrolyzed in 4 per cent sulfuric acid in a bath of boiling water and crystalline *d*-galactose isolated from the sugar syrup. This latter sugar thus appears to occupy one extreme of the molecule. Since *l*-rhamnose galacturonide has been identified among the products of hydrolysis of the mucilage, it appears that in this disaccharose uronide the uronic acid is combined with the pentose or methylpentose and the latter is combined with the hexose or methylated hexose. Fraction E thus represents the first step in the hydrolysis of the mixture of polyuronides which make up the mucilage.

TABLE II  
*Analysis of Calcium Salt, Fraction E, from Slippery Elm Mucilage*

	Fraction E before oxidation	Fraction E after oxidation
Calcium oxide, per cent.....	6.15	10.85
Carbon dioxide, per cent.....	9.20	8.44
<i>l</i> -Rhamnosan, per cent.....	18.00	16.50
Pentosan (xylan), per cent.....	4.50	3.50
Methoxyl, per cent.....		3.60
Aldehyde (CHO), per cent.....	8.00	0.00
Equivalent weight from CO <sub>2</sub> determination.....	477	521
$[\alpha]_D^{25}$ , degrees.....	+57.5	+41.5

#### SUMMARY

1. The mucilage has been prepared from the bark of slippery elm, *Ulmus fulva*, and shown to be a mixture of two or more polyuronides with varying amounts of calcium oxalate as an impurity.

2. The mucilage has been hydrolyzed and the presence of the following substances established: (a) galacturonic acid, (b) *l*-rhamnose, (c) *d*-galactose, (d) a colored non-saccharine X body. In addition there are indications of the presence of a pentose sugar, a methylated hexose sugar, and a methylated uronic acid.

3. The polyuronides of the mucilage seem to be composed of a uronic acid combined with 2 molecules of simple sugars and an X body in the following sequence. The uronic acid is combined through its aldehyde group with the methylpentose or pentose. The latter is combined through its aldehyde group with the hexose



or methylated hexose. Finally the hexose or methylated hexose is combined through its aldehyde group with the X body.

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## A MICROMETHOD FOR THE DETERMINATION OF URONIC ACIDS\*

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The original macromethod of Tollens and Lefevre (1) for the determination of uronic acids has undergone various modifications in the hands of different investigators (2-8). All of the modifications are based on the principle that the simple uronic acids and the complex polyuronide substances yield carbon dioxide quantitatively when distilled with 12 per cent hydrochloric acid. In some the carbon dioxide is determined gravimetrically, in others by titrimetric methods. The size of the sample used varies between the limits of 0.2 to 1.0 gm. and the period of heating from 4 to 8 hours.

Recently Buston (9) described a micromethod embodying titrimetric technique for the determination of uronic acid anhydride groups in pectic substances. In conjunction with the studies on the carbonyl sugar acids in progress in this laboratory, one of us (K. P. L.) and a collaborator<sup>1</sup> were engaged with the development of a satisfactory micromethod for their determination prior to the time of the appearance of Buston's method. The details had not been completely worked out, consequently it appeared advisable to evaluate the merits and defects of Buston's method in conjunction with our work.

Buston's method is open to criticism. The apparatus is very

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fragile and difficult to clean. We were also impressed by the fact that the method appeared to be supported by rather meager analytical data. The data were confined entirely to such complex substances as calcium pectate ( $C_{25}H_{46}O_{33}Ca_2$ ), sodium pectate ( $C_{25}H_{46}O_{33}Na_4$ ), and pectin, whose exact composition might still be considered an open question. With substances of such complexity the substitution of one or more residues of a pentose sugar by hexose residues or the loss of a methoxyl or acetyl residue, would alter but slightly the elementary composition. Thus the galacturonic acid anhydride figures obtained might approximate the values expected on the basis of the empirical formulæ presented, even though the complex molecules are actually not identical with these formulæ. Furthermore, within the limits of the analytical figures reported, considerable quantities of impurities might be present.

In the method described herewith the desirable features of Buston's procedure are employed along with certain parts of the excellent apparatus described by Clark (10) for the microdetermination of methoxyl groups.<sup>2</sup> With authentic preparations of *d*-glucuronic and *d*-galacturonic acid, various methoxyl derivatives of *d*-galacturonic acid, and highly purified polyuronide substances, the optimum temperature and the duration of the heating period necessary to effect complete decarboxylation were ascertained. It was found that when the decarboxylation is performed at a bath temperature of 133–136°, results in agreement with the theoretical values were obtained, provided the hydrolysis and decarboxylation period was conducted for a period of not less than 120 minutes. With authentic polygalacturonic acid anhydride preparations,  $(C_6H_5O_6)_n$ , the duration of the reaction had to be extended to 2½ hours. Highly purified specimens of alginic acid,  $(C_6H_5O_6)_n$ , required 3½ hours.<sup>3</sup>

It is well known that the accurate measurement of small quanti-

<sup>2</sup> The apparatus designed by Clark is a modification of the original micro-Zeisel apparatus developed by Pregl ((11) p. 199).

<sup>3</sup> This is in agreement with the experiences of Nelson and Cretcher (12), and those of Schoeffel and Link (13), who observed that in the microdetermination of the uronic anhydride content of alginic acid the time required for hydrolysis and decarboxylation had to be increased considerably in order to obtain consistent results.

ties of carbon dioxide by either gravimetric or titrimetric method presents formidable difficulties. The numerous factors involved have been thoroughly presented in the various treatises dealing with organic microanalysis (11, 14, 15), hence they need not be restated here.

We have explored both the gravimetric and titrimetric methods using the same conditions for the decarboxylation. Although we have been able to obtain consistent results with the titrimetric method presented below, we prefer the gravimetric method since it appears to be more accurate. It is also less tedious and more rapid. It should be emphasized that the titrimetric procedure is preferable when the temperature and atmospheric conditions are such as to make it difficult to obtain accurate weighings of the absorption tubes by the gravimetric method.

Since the gravimetric technique employed for the estimation of the carbon dioxide is identical with that developed by the late Professor Fritz Pregl for the determination of carbon in organic substances, the details need not be given. It should, however, be pointed out that accurate and reliable results cannot be realized unless the conditions originally prescribed by Pregl (11) and later by others (14-16) for the manipulation of the absorption tubes and the microbalance be *followed rigidly*.

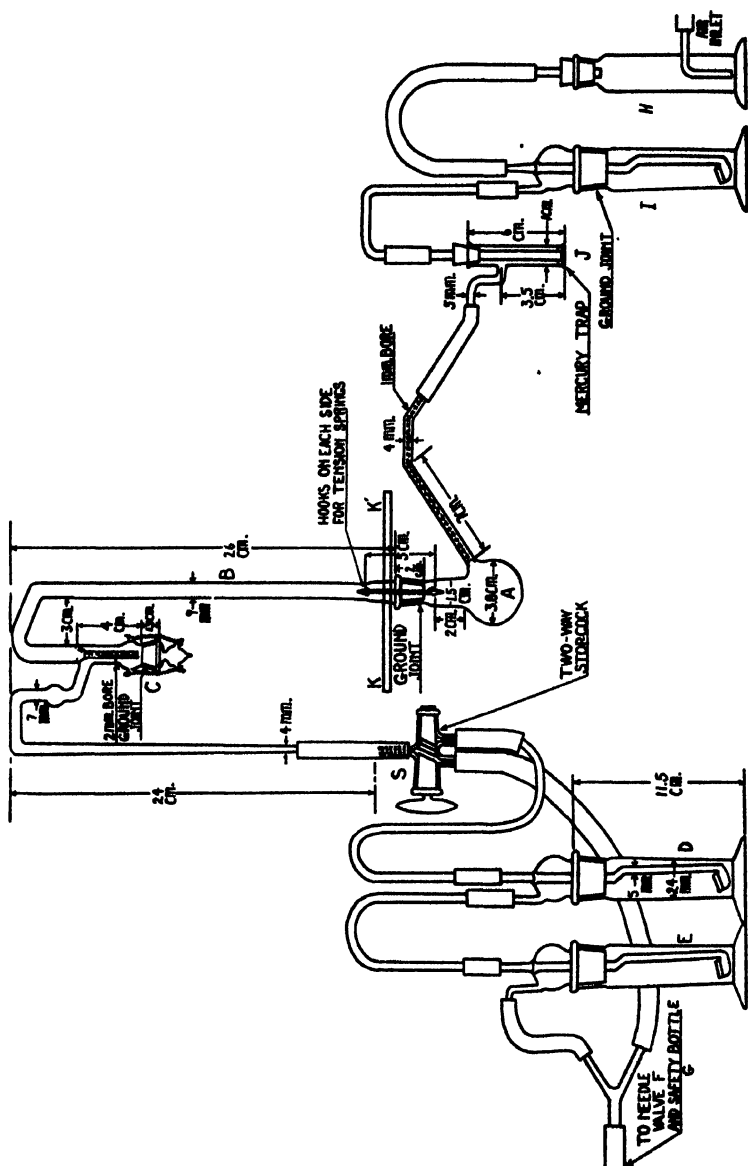
#### EXPERIMENTAL

*Description of Apparatus*—The apparatus consists of a reaction flask, *A*, with a capacity of 25 cc., which is connected to an air condenser, *B*, through a standard ground joint No. 6. The air condenser is 25 cm. long and terminates in a trap, *C*, containing silver sulfate dissolved in concentrated sulfuric acid, to retain any hydrogen chloride gas that might pass the condenser from the flask *A*. The joint in trap *C* is also standard No. 6. From trap *C* a side arm leads to the absorption tubes, or bottles *D* and *E*, through a 2-way stop-cock, *S*. The absorption system in the titrimetric method consists of small Jena gas wash bottles of 20 cc. total capacity, equipped with interchangeable ground glass stoppers carrying the inlet and outlet tubes. The inlet tube terminates in a Jena sintered glass disk (porosity No. 0) which breaks the gas stream into a spray of fine bubbles to insure efficient absorption. The bottles are connected to a filter pump through a

needle valve, *F*, which enables the rate of aspiration to be controlled accurately. A safety bottle, *G* (2.5 liter capacity equipped with a stop-cock), is shunted in to serve as a reservoir to equalize the flow of gas through the system. The aspirated air is freed from carbon dioxide by a train consisting of a small tower, *H*, filled with ascarite or soda-lime and a gas bottle, *I*, of the type described above, filled with saturated barium hydroxide solution. A small mercury trap, *J*, prevents the liberated  $\text{CO}_2$  from backing up into the purification train. The reaction flask *A* is placed in an oil bath which is heated with a microburner. The air condenser is protected from the heat of the bath with a piece of sheet asbestos, *KK'*, which is placed over the oil bath and around the condenser. The reaction flask should be immersed in the oil bath to such a depth that the levels of the reaction mixture in flask *A* and the outside oil are the same. The thermometer should not touch the sides of the bath. In order to promote smooth boiling a small boiling rod is placed in the flask. It consists of a piece of glass tubing, approximately 2 mm. internal diameter and 5 cm. long, sealed at one end and also about 5 mm. from the other end. The open end is fire-polished. The rod is introduced open end down. All the rubber connections should consist of properly treated and aged thick walled tubing of the type used in the Pregl carbon and hydrogen microdeterminations.

*Analytical Procedure for Titrimetric Method*—A sample of 9 to 11 mg. is weighed out accurately in a boat made from about one-third of a cigarette paper. The paper boat is folded carefully and introduced into the reaction flask.<sup>4</sup> 7 cc. of 12 per cent  $\text{HCl}$  saturated with sodium chloride are then added, the boiling rod introduced, and the flask connected to the apparatus. The trap *C* is previously filled with a few drops of concentrated sulfuric acid containing silver sulfate. The 2-way stop-cock is set to establish a direct connection to the needle valve. The air in the apparatus is then removed by drawing a small but steady stream of  $\text{CO}_2$ -free air through the system. This requires about 10 minutes. Meanwhile 20 cc. of 0.02 *N*  $\text{Ba}(\text{OH})_2$  are added to each of the gas bottles *D* and *E* which are then connected to the apparatus with

<sup>4</sup> Blank determinations on the cigarette paper used showed that no appreciable quantities of  $\text{CO}_2$  are produced by the action of the hydrochloric acid.



**Fig. 1. Apparatus for the microdetermination of uronic acids and polyuronide substances**

thick walled tubing as shown in Fig. 1. The heating is now started without altering the initial rate of aspiration. (A small burner is convenient for this purpose.) When the temperature of the oil bath reaches 100° the stop-cock *S* is turned so that the air stream passes through the gas bottles. As the temperature approaches 120° care must be taken to maintain sufficient aspiration to prevent excessive back pressure on mercury trap *J*. The contents of the flask begin to boil at approximately 120° and equilibrium is soon reached. The burner is adjusted so that the temperature of the oil bath is maintained between the limits of 135–137°. The aspiration is adjusted so that a small, steady stream of bubbles passes through the gas bottles. At the end of 2 hours or a longer period, depending on the substance, the heating is discontinued and the stop-cock *S* is turned to its former position. The gas bottles *D* and *E* are disconnected, the ground glass heads replaced by rubber stoppers, and the barium carbonate allowed to settle.

The titration of the barium hydroxide solution from the gas wash bottles should always be preceded by the following method of standardizing the relative strength of the acid and the alkali solutions. Approximately 20 cc. of the standard barium hydroxide are withdrawn from the burette into a small flask (25 cc.). By means of a standardized 5 cc. Ostwald pipette, two aliquots are then rapidly pipetted into 125 cc. Erlenmeyer flasks containing 20 cc. of carbon dioxide-free water and 3 drops of phenolphthalein. The aliquots are then titrated with 0.01 *N* sulfuric acid. Not more than two aliquots should be pipetted at one time. The aliquots should agree within 0.04 cc. of 0.01 *N* acid. These titrations give the acid-alkali ratio which must be determined each time a sample is analyzed, since it may vary slightly from time to time. In exactly the same manner two 5 cc. aliquots from each of the gas bottles *D* and *E* are then titrated.

The barium hydroxide solution is accurately standardized once each week with analytically pure potassium acid phthalate.

The calculations are made as follows:

Let *A* equal cc. 0.01 *N* acid equivalent to 5 cc. alkali

" *B* " " 0.01 " " " 5 " aliquots of bottle *D*

" *C* " " 0.01 " " " 5 " " " *E*

Each gas bottle contains 20 cc. of standard alkali. 1 cc. of 0.01 *N*  $\text{H}_2\text{SO}_4$  is equivalent to 0.00022 gm. of  $\text{CO}_2$ ; therefore,

$$\frac{4((A-B) + (A-C))(0.00022)(\text{acid factor})(100)}{\text{Weight of sample}} = \text{per cent } \text{CO}_2$$

The following representative calculation is based on figures obtained in a determination of the uronic acid content of methyl-*d*-galacturonide dihydrate,  $C_7H_{12}O_7 \cdot 2H_2O$ .

*Acid-Alkali Ratio*—5 cc. of 0.02 *N* Ba(OH)<sub>2</sub> (factor = 1.005) required 9.45 cc. of 0.01 *N* sulfuric acid. Therefore the 0.01 *N* H<sub>2</sub>SO<sub>4</sub> has a factor of 1.050.

*Titration of Aliquots from Bottles D and E*

Two 5 cc. aliquots from bottle <i>D</i>	= 7.38
	<u>7.38</u>
	7.38 cc., average
Two 5 cc. aliquots from bottle <i>E</i>	= 9.35
	<u>9.34</u>
	9.345 cc., average

*Calculation*

$$\frac{4((9.45 - 7.38) + (9.45 - 9.34))(0.00022)(1.05)(100)}{0.01119 \text{ gm.}} = 18.00 \% \text{ CO}_2$$

The theoretical for the methylgalacturonide dihydrate is 18.03 per cent.

Achromatic indicators described by Smith (17) may be used in place of phenolphthalein as suggested in Buston's article (9). Such indicators were tried but we feel that they offer only slight, if any, advantages over phenolphthalein.

*Analytical Procedure for Gravimetric Modification*—In the gravimetric method the standard Pregl soda-lime absorption tubes ((11) p. 45) are used to collect the carbon dioxide liberated. A Schwartz U-tube equipped with ground glass stoppers, containing concentrated sulfuric acid and a few glass beads is connected directly to the side arm of the decarboxylation apparatus in place of the 2-way stop-cock *S*. The other outlet of the U-tube is connected to a second Schwartz U-tube containing dehydrite (18) or porous anhydrous calcium chloride (groat size) as prescribed by Pregl ((11) p. 54). The first U-tube contains just enough concentrated sulfuric acid to cover the beads in the bottom of the tube. The flow of the gas bubbles through this tube should be about 18 to 24 per minute.

The 2-way stop-cock *S* is next, being joined to the side arm of the second U-tube containing the dehydrite or the calcium chloride. The Pregl soda-lime tubes follow; they are connected to one of the limbs of the stop-cock *S*. The Pregl tubes are connected with the



regular thick walled rubber tubing (8 mm. outer diameter, 2 mm. bore) treated as prescribed for the carbon and hydrogen microdetermination ((11) pp. 54-57). A Pregl calcium chloride tube with two connecting tubes bent at right angles ((11) p. 54) is placed between the second soda-lime absorption tube and one limb of the Y-tube that leads to needle valve *F*. The other limb of the Y-tube is connected to the remaining limb of stop-cock *S*. This tube prevents the second Pregl soda-lime tube from absorbing water from the aspiration system. The other aspects of the procedure used in the gravimetric method are comparable to the details given above for the titrimetric method. It should be reemphasized that the soda-lime tubes should be manipulated as prescribed for the Pregl carbon and hydrogen microdetermination ((11) pp. 43-53, 80-87).

*Sources of Errors*—The micromethod presented is recommended *only for pure uronic acids or their derivatives*. Other carbohydrate substances like the sugars, starch, cell wall polysaccharides, and certain organic acids yield small quantities of carbon dioxide when boiled with 12 per cent hydrochloric acid. While the quantity of carbon dioxide liberated from these substances frequently does not interfere with the accuracy of the macromethods (7), the same is not necessarily true in the microdetermination. Consequently the method is not recommended for uronic acid determinations on plant extracts or crude polysaccharide preparations containing small quantities of uronic acids. The most important sources of error in the uronic acid microdetermination are the rate of aspiration and the length of the heating period. Low results are invariably obtained when the rate of aspiration is too rapid and when the heating period is too brief. A very slow aspiration accompanied with the existence of an occasional back pressure in the course of the determination can likewise produce low results.

The titrimetric modification is obviously subject to the various sources of error that accompany the handling of dilute standard solutions. Consequently the standard precautions recommended for titrimetric work must be rigidly observed. With the gravimetric modification, the commonest source of error is invariably due either to the improper handling of the absorption tubes or to adverse temperature and atmospheric conditions that influence the constancy of the absorption tubes and the microbalance. The

TABLE I  
Summary of Analytical Results

Substance	Size of sample mg.	Length of heating hrs.	Per cent carbon dioxide			
			Calcu- lated	Found by gravimetric method	Found by titrimetric method	Found by titrimetric method
<i>d</i> -Galacturonic acid monohydrate, $C_6H_{10}O_7 \cdot H_2O$ ; m.p. 159; $[\alpha]_D = +53.4^\circ$	10	2	20.75	20.88	20.85	20.90
<i>d</i> -Glucuronic acid, $C_6H_{10}O_7$ ; m.p. 163; $[\alpha]_D = +34.0^\circ$	10	2	22.68	22.10	21.98	22.34
Methyl- <i>d</i> -galacturonide dihydrate, $C_7H_{12}O_7 \cdot 2H_2O$ ; m.p. 112-114; $[\alpha]_D = +127.6^\circ$	10	1½	18.03	18.34	18.26	18.00
Methyl- <i>d</i> -galacturonide methyl ester monohydrate, $C_8H_{14}O_7 \cdot H_2O$ ; m.p. 140-141; $[\alpha]_D = +124.1^\circ$	10	2½	18.33	17.80	18.65	18.12
Methylglycoside methyl ester polygalacturonide, $C_6H_8O_7COOH$ , $(C_6H_7O_7COOCH_3)_n$ , $C_6H_7O_7(OCH_3COOCH_3)_n$ ; $[\alpha]_D = +198^\circ$	10	2	22.21	22.30	22.99	22.59
Alginate acid, $(C_6H_7O_6)_n$ ;† $[\alpha]_D = -136.0^\circ$	10	3	25.00	24.30	24.05	24.45
Polygalacturonide, $(C_6H_7O_6)_n$ ;† $[\alpha]_D = +259.0^\circ$	10	2½	25.00	25.31	24.39	25.21
						22.23
						22.81
						23.93
						24.28
						24.17
						24.51

\* Prepared from a polygalacturonide isolated from citrus pectin after the method of Morell and Link (19).

† Results calculated on ash- and methoxyl-free basis.

sources of these errors have been fully described in the handbooks dealing with the carbon and hydrogen microdetermination (11, 14-16). The various substances used in the absorption and gas washing trains must obviously be changed at frequent intervals in order that fresh effective reagents are always at hand. The first Pregl soda-lime tube can be used for seven to eight determinations—the second tube can be safely used for twelve to fifteen determinations.

#### SUMMARY

1. A micromethod with either a volumetric or gravimetric modification is given for the accurate determination of uronic acids and uronic acid derivatives by decarboxylation with hydrochloric acid (sp. gr. 1.06).

2. The results obtained with authentic specimens of *d*-galacturonic acid, *d*-glucuronic acid, methyl-*d*-galacturonide dihydrate, methyl ester methyl-*d*-galacturonide monohydrate, a methylglycoside methyl ester polygalacturonide,  $C_6H_5O_5COOCH_3$  ( $C_6H_7O_4COOCH_3$ )<sub>n</sub>,  $C_6H_7O_5$  ( $OCH_3$ )  $COOCH_3$ , a pure polygalacturonide,  $(C_6H_5O_5)_n$ , and alginic acid,  $(C_6H_5O_6)_n$ , are presented (Table I).

3. In all cases, the results obtained by either the volumetric or the gravimetric modification approximate the theoretical values.

4. The micromethod presented is capable of giving results of the same order of accuracy as the best macromodifications.

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# THE METHYLGLYCOSIDES OF THE NATURALLY OCCURRING HEXURONIC ACIDS\*

## II. THE KINETICS OF THE HYDROLYSIS OF $\alpha$ -METHYL-*d*-GALACTURONIDE

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It has been generally assumed that the ring structure of the aldehyde sugar acids, as well as their naturally occurring polymers, is analogous to that of the parent sugars. Haworth and coworkers, in their extensive studies of the saccharides, have shown that two distinct groups exist, the pyranosides and the furanosides (1). The evidence for this classification was partly based on the relative rates at which the two types of glycosides were hydrolyzed in acid solutions. The velocity constants for the furanosides were approximately 100 to 200 times greater than the respective pyranosides (2). Since the pyrane ring predominates in the simple sugar group, it has become customary to assign the same ring system to the common naturally occurring hexuronic acids, *d*-glucuronic, *d*-mannuronic, and *d*-galacturonic acids (3). The validity of this assumed parallelism has been corroborated thus far only in the case of *d*-glucuronic acid as it occurs in the aldobionic acid from gum arabic (4), in bornyl-*d*-glucuronide (5), and in euxanthic acid (6).

The isolation of  $\alpha$ -methyl-*d*-galacturonide has made it possible to study the ring structure of the naturally occurring *d*-galacturonic acid.<sup>1</sup> The stability of this isomer indicated that it probably possessed the pyranoid ring.

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<sup>1</sup> The evidence presented in preceding papers on methyl-*d*-galacturonide (7, 8) indicating that the compound isolated was the  $\alpha$  isomer has been confirmed independently by Smolenski and Cichocki (9) and by Ehrlich and Guttman (10). These investigators succeeded in isolating the  $\beta$  as well as the  $\alpha$  form.

## 184 Methylglycosides of Hexuronic Acid. II

A very extensive study of the kinetics of glycoside hydrolysis has been made by Moelwyn-Hughes who investigated the behavior of some twenty compounds at different temperatures (11). All of the reactions were pseudounimolecular, following the equation:

$$k = \frac{1}{t} \ln \frac{C_0}{C_t} \quad (1)$$

where  $k$  = velocity constant;  $t$  = time interval;  $C_0$  = initial concentration;  $C_t$  = concentration at  $t$ . The velocity constants were dependent on both temperature and pH (11, 12). Moelwyn-Hughes emphasized the importance of measuring the temperature coefficients for these reactions, particularly in connection with any comparisons of acidic and enzymatic hydrolyses, since these must necessarily be investigated at different temperatures.

The velocity constants obtained for the hydrolysis of  $\alpha$ -methyl-*d*-galacturonide, reported below, show that this compound undoubtedly belongs to the group containing the stable amylene oxide ring. Since  $\alpha$ -methyl-*d*-galactoside was not included in the investigations of Moelwyn-Hughes, it was of interest to measure its constants and compare them to the related galacturonide. It was found that the values of  $k$  were practically identical, from which it may be concluded that the same ring type is contained in both compounds. Since the ring structure of  $\alpha$ -methyl-*d*-galactoside is known beyond doubt,  $\alpha$ -methyl-*d*-galacturonide may therefore be included among the stable pyranoside derivatives.

### *Experimental Procedure and Results*

*Preparation and Physical Constants of Substances Studied*—The  $\alpha$ -methyl-*d*-galacturonide dihydrate used in the following experiments was prepared from commercial pectic acid by the procedure of Morell and Link (8). It was twice recrystallized from 95 per cent ethyl alcohol and melted at 112°, decomposing sharply at 120°; it showed a rotation of  $[\alpha]_D^{25} = +127.8^\circ$  in a 2.5 per cent water solution. The  $\alpha$ -methyl-*d*-galactoside, prepared from a pure specimen of *d*-galactose by the Fischer procedure (13), was also recrystallized twice from 95 per cent ethyl alcohol. When heated for 19 hours at 100° over phosphorus pentoxide at 5 mm. pressure, it lost 8.30 per cent of its weight, whereas theory requires 8.50 per cent for the monohydrate. The dried sample melted at

114–116° and showed a rotation of  $[\alpha]_D^{25} = +199.4^\circ$  in a 1.4 per cent water solution.

*Procedure for Determining the Hydrolysis of  $\alpha$ -Methyl-*d*-Galacturonide and  $\alpha$ -Methyl-*d*-Galactoside*—The hydrolysis of the glycosides to the corresponding free carbonyl compounds was followed by means of Goebel's modification of the Willstätter-Schudel hypiodite titration (14). Since mineral acids degrade the uronic acids to *furanoid* substances (15), the reducing values for the completed reactions were calculated. All of the titrations were made before any noticeable degradation occurred. An authentic specimen of *d*-galacturonic acid was found to react quantitatively with the Willstätter-Schudel reagent. This indicated that the above titrations served as an accurate measurement of the extent of hydrolysis. In measuring the constants for  $\alpha$ -methyl-*d*-galacturonide a slight increase in  $k$  was always observed, particularly for the last two or three points. This indicates that the degradation of the free galacturonic acid formed during the hydrolysis is associated with an increasing reducing power. Due to this complicating factor, the simple method of taking the arithmetical mean of the observed values was most practicable, although for a more extensive study of this monomolecular reaction the methods of analysis described by Guggenheim (16) and by Roseveare (17) should be used. For this investigation, however, the slight drift observed is insignificant, since any difference in ring structure between  $\alpha$ -methyl-*d*-galactoside and  $\alpha$ -methyl-*d*-galacturonide would make a difference of several hundredfold in the values of  $k$ . In all of the experiments the following procedure was employed.

A solution 0.1 *N* in glycoside and *N* in hydrogen chloride concentration was prepared by dissolving 3.050 gm. of  $\alpha$ -methyl-*d*-galacturonide dihydrate, or 2.650 gm. of  $\alpha$ -methyl-*d*-galactoside monohydrate, in 9 cc. of water and diluting to 250 cc. in a volumetric flask with a 1.038 *N* hydrochloric acid solution. After thorough mixing, two 5 cc. aliquots were removed and titrated with an approximately *N* sodium hydroxide solution. The remainder was transferred to a 500 cc. flask (preferably 3-necked so as to facilitate subsequent withdrawal of samples) equipped with a condenser and immersed in a thermostat previously regulated to the desired temperature. After about 30 minutes, the first sample was withdrawn (in duplicate) by means of a 10 cc. pipette, which had been stand-



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ardized at the temperature of the reaction mixture, and transferred to a 250 cc. Erlenmeyer flask containing the exact amount of sodium hydroxide solution required to neutralize the sample. When the pipette had drained, the time interval was recorded. 20 cc. of 0.1 N I<sub>2</sub>-KI solution were then added, followed by 35 cc. of 0.1 N sodium hydroxide solution which was added in drops with constant shaking during the course of 3 minutes. (It is particularly important to add the first 10 cc. of alkali slowly.) The

TABLE I  
*Velocity of Hydrolysis of  $\alpha$ -Methyl-d-Galacturonide and  $\alpha$ -Methyl-d-Galactoside in N HCl at 70°*

$\alpha$ -Methyl-d-galacturonide			$\alpha$ -Methyl-d-galactoside		
<i>t</i>	Thio	$k \times 10^3$	<i>t</i>	Thio	$k \times 10^3$
min.	cc.	min. <sup>-1</sup> *	min.	cc.	min. <sup>-1</sup>
0	19.31		0	19.08	
114	17.84	1.48	30	18.53	2.05
182	17.03	1.52	73	17.80	2.05
287	15.93	1.54	231	15.65	2.01
376	15.10	1.57	332	14.62	1.99
467	14.38	1.51	507	13.21	2.00
587	13.51	1.62	650	12.40	1.99
806	12.34	1.68	827	11.63	2.00
Infinite	9.86 (calculated)		Infinite	9.86 (calculated)	
Average $k = 1.56 \times 10^{-3} \text{ min.}^{-1}$			Average $k = 2.01 \times 10^{-3} \text{ min.}^{-1}$		

10 cc. pipette = 9.86 cc.; factor 0.1 N thiosulfate = 1.001; 20 cc. 0.1 N I<sub>2</sub>-KI (pipette) = 19.70 cc. thiosulfate.

\* min.<sup>-1</sup> means that the values for  $k$  are expressed in reciprocal minutes.

solution was diluted with water to 100 cc. and the flask stoppered. After standing at room temperature for 15 minutes, the solution was acidified with about 5 cc. of 10 per cent hydrochloric acid and titrated with 0.1 N thiosulfate solution.

As an example of the method, the following data obtained for each compound hydrolyzed at 70° is presented (Table I). The values of  $k$  were calculated by using the following modification of Equation 1.

$$k = \frac{2.303}{t} \log_{10} \frac{(\text{cc. thiosulfate at } t_0) - (\text{cc. thiosulfate at } t_\infty)}{(\text{cc. thiosulfate at } t) - (\text{cc. thiosulfate at } t_\infty)}$$

*Calculation of Critical Increments for Hydrolysis of  $\alpha$ -Methyl-d-Galacturonide and  $\alpha$ -Methyl-d-Galactoside*—From the Arrhenius equation (18)

$$\frac{d \ln k}{dT} = \frac{E}{RT^2} \quad (2)$$

where  $k$  = velocity constant,  $R$  = molar gas constant,  $T$  = absolute temperature,  $E$  = critical increment, it is possible to calculate the critical increment (sometimes called the energy of activation) for the hydrolysis. In order to apply this equation, the hydrolysis of  $\alpha$ -methyl-d-galacturonide was measured at 60° and at 80°. The

TABLE II  
*Effect of Temperature on Velocity of Hydrolysis of  $\alpha$ -Methyl-d-Galacturonide in N HCl*

$T$	$k \times 10^3$	$\log k$	$-R(2.303)\log k$	$1/T$
	min. <sup>-1</sup>			
353	5.42	7.7340-10	10.38	0.002832
343	1.56	7.1931-10	12.85	0.002916
333	0.448	6.6513-10	15.33	0.003003

value for  $E$  should be constant between any two given temperatures, and a straight line obtained when  $(-R \ln k)$  is plotted against the reciprocal of the absolute temperature. Such a graph for  $\alpha$ -methyl-d-galacturonide may be obtained from the values of  $k$  at 60°, 70°, and 80°, along with the calculations which are given in Table II. The points fall on a straight line and the slope of the curve gives the value of  $E$  directly, which is found to be 29,000 calories. From such a curve it is possible to calculate the velocity of hydrolysis for  $\alpha$ -methyl-d-galacturonide in N hydrochloric acid solutions at any temperature. It is furthermore possible to calculate  $k$  at any pH for Moelwyn-Hughes (11) and Bolin (12) have shown that a linear relationship exists between the velocity constant and the hydrogen ion activity.

$\alpha$ -Methyl-d-galactoside possesses a slightly higher critical increment than  $\alpha$ -methyl-d-galacturonide. At 80° the former showed a velocity constant of  $k = 8.7 \times 10^{-3}$  minutes<sup>-1</sup>, and by substitution of this value, along with that of  $2.01 \times 10^{-3}$  minutes<sup>-1</sup>, obtained at 70°, in Equation 2, a solution for  $E$  gives 35,000 calories. By calculating the point of intersection on the graph for both

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compounds studied, it may be shown that at 69.6° the velocity of hydrolysis of  $\alpha$ -methyl-*d*-galacturonide is identical to that of  $\alpha$ -methyl-*d*-galactoside.

### SUMMARY

The velocity of hydrolysis of  $\alpha$ -methyl-*d*-galacturonide has been measured at three different temperatures in *N* hydrochloric acid solutions and compared with the constants of  $\alpha$ -methyl-*d*-galactoside obtained under identical conditions. The critical increments for both reactions were calculated by means of the Arrhenius equation. It was found that the velocity constants for both compounds were practically identical and that the  $\alpha$ -methyl-*d*-galactoside possessed a slightly higher critical increment. It may be concluded that  $\alpha$ -methyl-*d*-galacturonide contains a six-membered ring. These observations lend further support to the contention that the stable forms of the hexuronic acids are pyranoside derivatives.

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## SYNTHESIS OF THE HEXURONIC ACIDS\*

### III. THE SYNTHESIS OF *dl*-ALLURONIC ACID FROM ALLOMUCIC ACID

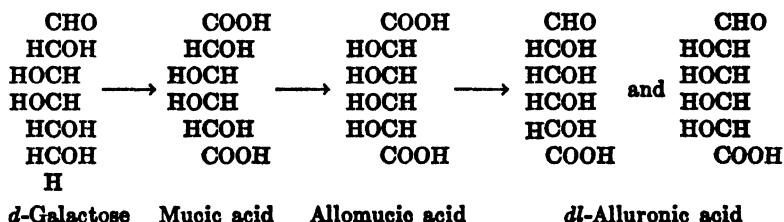
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All of the aldohexuronic acids which have thus far been synthesized are related to naturally occurring aldohexoses. *d*-Glucuronic, *d*-mannuronic, and *dl*-galacturonic acids have been synthesized by the partial reduction of the mono- or dilactones of the corresponding dicarboxylic sugar acids (1-3). Furthermore, *d*-galacturonic acid has been obtained by the oxidation of diacetone-*d*-galactose (4, 5). In this communication we report the synthesis of *dl*-alluronic acid, a member of the galactose series.

The reactions involved in the synthesis are represented by the following structural formulæ.



#### EXPERIMENTAL

The technique employed in this synthesis is essentially the same as that described by Fischer for the synthesis of *d*-glucuronic acid (1) and more recently by the authors for the synthesis of *d*-man-

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nuronic acid (2).<sup>1</sup> Exact details are given only when the procedure has not been fully described before.

The melting points (uncorrected) reported in this communication were determined in the Thiele apparatus equipped with an enclosed scale thermometer. In all cases the temperature of the bath was raised at the rate of 6° per minute.

All the elementary analyses were made with the Pregl micro-methods.

*Preparation of Allomucic Acid*—Crystalline allomucic acid was prepared by the epimerization of mucic acid following the conditions described by Posternak and Posternak (6). The crude product obtained from the epimerization reaction was recrystallized three times from hot water as recommended by Fischer (7). The purified acid melted at 170–172°.

*Preparation of Barium dl-Alluronate*—The acid was converted into its lactone by dissolving 1 part in 60 parts of hot water and rapidly evaporating the resulting solution over a free flame to 0.1 of its original volume. The lactone solution was reduced with 2.5 per cent sodium amalgam following the procedure of Fischer (1) and Niemann and Link (2). After the inorganic salts and the unchanged allomucic acid had been removed the *dl*-alluronic acid was converted into its barium salt by the usual procedure (2) and isolated in this form. The yield of barium *dl*-alluronate from 25 gm. of allomucic acid was approximately 4 gm.

<i>Analysis</i> —Calculated for $(C_6H_7O_7)_2Ba$ .	Ba	26.30
Found.	"	26.26

*Preparation of dl-Alluronic Acid*—During the course of 30 minutes, 17.00 cc. of *N* sulfuric acid were added to a solution of 5.0 gm. of barium *dl*-alluronate in 15 cc. of water. After all the acid had been added, 250 cc. of 95 per cent ethyl alcohol were introduced into the reaction mixture, which was then placed in the

<sup>1</sup> In the first paper of this series on the synthesis of *dl*-galacturonic acid we described a technique for the isolation of *dl*-galacturonic acid wherein we used basic lead acetate. Experience has shown that it is inferior to the conditions originally employed by Fischer (1). Consequently in the synthesis of *d*-mannuronic acid (2) and *dl*-alluronic acid we employed the conditions described by Fischer for the synthesis of *d*-glucuronic acid (1). This improvement has also been used in additional studies conducted by us on the synthesis of *dl*-galacturonic acid.

ice chest overnight. The insoluble barium salts were filtered off and the solution concentrated under diminished pressure to 20 cc. The concentrate was poured into 100 cc. of absolute ethyl alcohol and cooled in an ice bath for 4 hours. The precipitated barium *dl*-alluronate was filtered off and the filtrate concentrated under reduced pressure to a volume of 5 cc. 200 cc. of absolute ethyl alcohol were added to the concentrate and the resulting solution placed in the ice chest overnight. The last traces of the precipitated barium *dl*-alluronate were removed by filtration. The clear filtrate was then concentrated to a volume of 15 cc. and filtered into a crystallizing dish. The dish was placed in a desiccator containing calcium chloride and allowed to stand for several days in the ice chest. The contents of the dish soon turned into a solid cake which was collected after trituration with 10 cc. of absolute ethyl alcohol. The product (1.50 gm.) had a slight pink color and contained considerable quantities of the lactone as an impurity. The acid was separated from its lactone by two recrystallizations from hot absolute ethyl alcohol. The *dl*-alluronic acid was finally obtained as a white microcrystalline powder. When crystallized under the conditions reported above, *dl*-alluronic acid does not contain water of hydration, and in this respect it resembles the  $\beta$  form of *d*-galacturonic acid (8, 9). For analysis the compound was dried for 10 hours under reduced pressure over phosphorus pentoxide at 78°.

**Melting Point**—The acid melted at 145–146° and decomposed at 148–149°.

**Rotation**— $[\alpha]_D^{26} = 0.0^\circ$  (in water,  $c = 1.0$  per cent).

**Analysis**

Calculated for  $C_6H_{10}O_7$ . C 37.13, H 5.16, N.E.\* 51.52 cc. 0.1 N alkali  
Found. " 37.24, " 5.57, " 51.00 " 0.1 " "

\* N.E. represents the neutralization equivalent.

**Preparation of Brucine Alluronate**—2.50 gm. of barium *dl*-alluronate were converted into the brucine salt by the procedure described by Niemann and Link for the preparation of brucine *d*-mannuronate (2). The alkaloidal salt was purified by two recrystallizations from 80 per cent ethyl alcohol. By this procedure 1.50 gm. of brucine alluronate were obtained. The derivative when dried at room temperature over phosphorus pentoxide under

atmospheric pressure for 1 week contains 1 molecule of water which is lost by drying at 78° for 10 hours. The values reported for the melting point and rotation were observed on preparations that had been recrystallized (eight times) from 80 per cent ethyl alcohol until the rotation was constant. The values probably represent those of the brucine salt of one enantiomorph and not of the *dl* mixture.

*Melting Point*—The hydrate melts at 172–173° and the anhydrous salt melts at 180–181°.

*Rotation*— $[\alpha]_D^{28} = -25.0^\circ \pm 1.0^\circ$  (in water,  $c = 1.5$  per cent).

*Analysis*

Calculated for  $C_{12}H_{18}O_{11}N_2 \cdot H_2O$ . N 4.65,  $H_2O$  3.00,  $OCH_3$  10.30

Found. " 4.75, " 3.30, " 10.55

*Preparation of Barium p-Bromophenylosazone-dl-Alluronate*—A mixture of 1.40 gm. of barium *dl*-alluronate, 4.10 gm. of *p*-bromophenylhydrazine hydrochloride, 6.00 gm. of barium acetate, 5 cc. of glacial acetic acid, and 100 cc. of water was treated according to the Goldschmiedt and Zerner procedure (10). The crude product obtained from the reaction mixture was extracted with ethyl ether, absolute ethyl alcohol, and dried at 60° over phosphorus pentoxide under diminished pressure. The compound (1.70 gm.) was obtained as a light yellow microcrystalline powder.

*Melting Point*—The derivative melted at 209–211° with decomposition.

*Analysis*

Calculated for  $(C_{12}H_{11}O_8N_4Br_2)_2Ba$ . Ba 11.49, N 9.37

Found. " 11.43, " 9.67

*Preparation of Methyl-dl-Alluronide Methyl Ester*—4.5 gm. of a pure syrup of *dl*-alluronic acid were treated in the usual manner (11) with anhydrous methyl alcohol and hydrogen chloride. After the hydrochloric acid had been removed with silver carbonate, the methyl alcohol was replaced with 95 per cent ethyl alcohol. On standing for 4 weeks (ice chest) the methyl-*dl*-alluronide methyl ester (0.06 gm.) crystallized out as the monohydrate. In its water of hydration content it is similar to the corresponding derivative of *d*-galacturonic acid (12–14).

*Analysis*

Calculated for  $C_8H_{14}O_7 \cdot H_2O$ .  $OCH_3$  25.84, S.E.\* 41.60 cc. 0.1 N alkali

Found. " 25.90 " 44.00 " 0.1 " "

\*S.E. represents the saponification equivalent.

## DISCUSSION

The rule of van't Hoff predicts the existence of sixteen isomeric aldohexuronic acids provided that the structure is assumed to be of the free aldehyde form. Of these sixteen isomers we can select eight that are individually enantiomorphs of the remaining eight configurations. Thus there are eight diastereoisomers, each having an enantiomorph. Of these eight possible diastereoisomers, four can be considered to be of the glucose series and four of the galactose series (15).

The two diastereoisomers of the galactose series, *e.g.* *dl*-galacturonic and *dl*-alluronic acids, resemble each other in several important respects. Both are known only as the crystalline free acids (9) and in both instances a crystalline lactone has not been obtained. On the other hand the two diastereoisomers of the glucose series, *e.g.* *d*-glucuronic and *d*-mannuronic acids, have been obtained as crystalline lactones (1, 9, 16, 17) and also as crystalline free acids (18, 19). The same relationships are encountered when the dibasic acids of the corresponding configurations are considered. In the galactose series mucic and allomucic acids have been obtained crystalline only as the free acids (8, 9). However, in the glucose series *d*-saccharic and *d*-mannosaccharic acids have been isolated in the form of the crystalline lactones (8, 9) and also as the crystalline free acids (8, 20).

*dl*-Alluronic acid shows all of the chemical reactions characteristic for the group of aldohexuronic acids (9, 21, 22). Barium *dl*-alluronate reacts with *p*-bromophenylhydrazine under the conditions described by Goldschmiedt and Zerner (10) to form a well defined barium *p*-bromophenylosazone-*dl*-alluronate. It has been shown previously that barium *d*-mannuronate and barium *d*-galacturonate form hydrazones when the Goldschmiedt and Zerner conditions are employed (23). Barium *d*-glucuronate, however, forms the osazone under the same conditions (10). The relationship between configuration and tendency toward hydrazone or osazone formation can be expressed as follows: when the configuration on carbon atoms (2), (3), and (4) in the aldohexuronic acid is symmetrical then its barium salt will form an osazone when the Goldschmiedt and Zerner conditions are employed; if the configuration is asymmetrical the hydrazone is formed. This empirical generalization is made on the basis of information gained from one-half of the theoretically possible configurations.



## SUMMARY

Crystalline *dl*-alluronic acid has been prepared by the reduction of allomucic acid lactone with sodium amalgam. The brucine salt, the methyl ester methylglycoside, and the barium salt of the *p*-bromophenylosazone of *dl*-alluronic acid have been prepared. Several relationships existing between the stereochemical configuration and the properties of the known aldohexuronic acids have been pointed out.

We are indebted to our colleague, Dr. Eugene Schoeffel, for conducting the microanalytical determinations.

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4

**SYNTHESIS OF THE HEXURONIC ACIDS\***  
**IV. THE SYNTHESIS OF *d*-GALACTURONIC ACID FROM**  
***d*-GALACTOSE**

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(Received for publication, October 9, 1933)

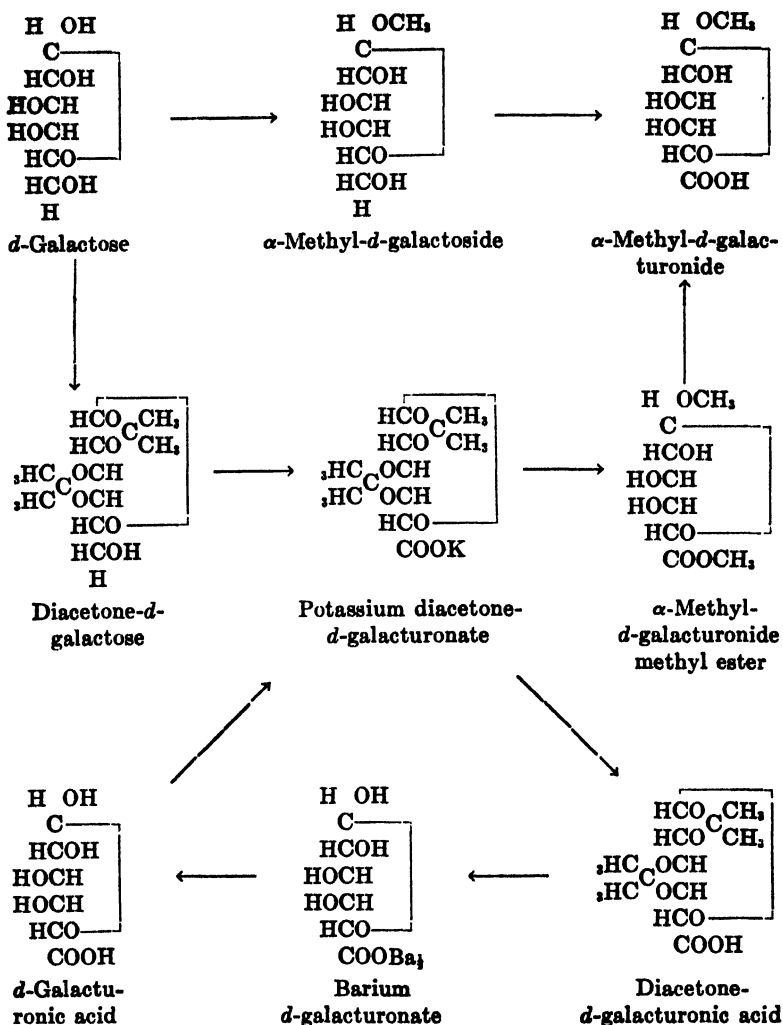
Diacetone-*d*-galactose yields on oxidation with potassium permanganate diacetone-*d*-galacturonic acid and oxalic acid (1, 2). Svanberg (1), the first to investigate this reaction, did not isolate the diacetone-*d*-galacturonic acid but determined its presence by the method of Tollens and Lefevre (3). Later Ohle and Berend (2) isolated diacetone-*d*-galacturonic acid and its potassium salt in a crystalline condition but they were unable to convert the diacetone compound to the crystalline *d*-galacturonic acid.

Through the application of a technique previously used in this laboratory for the isolation of aldohexuronic acids (4-7), we have been able to obtain crystalline *d*-galacturonic acid from the diacetone derivative in good yields. Further we succeeded in converting diacetone-*d*-galacturonic acid to the  $\alpha$ -methyl-*d*-galacturonide. The  $\alpha$ -methyl-*d*-galacturonide was identical with the glycuronide prepared by the oxidation of  $\alpha$ -methyl-*d*-galactoside and with the glycuronide obtained by the methylation of *d*-galacturonic acid isolated from natural sources (8-10).

The reactions involved in these transformations can be structurally represented as follows:

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Supported in part by grants from the University Research Fund.



## EXPERIMENTAL

The melting points reported herewith were determined in the Thiele apparatus equipped with an inclosed scale thermometer. The temperature of the bath was raised at the rate of 5–6° per minute. The rotations were observed with a Schmidt and Haensch (Berlin) quartz wedge saccharimeter equipped with the

Ventzke scale. The new electric sodium lamp made by the same firm was used as the source of light. In all cases the preparations used for the analyses (Pregl micromethods) were dried at room temperature over phosphorus pentoxide for 1 week.

*Preparation of Diacetone-d-Galactose*—Diacetone-d-galactose was prepared by following the sulfuric acid technique described by Ohle and Berend (2) and Ohle and Koller (11). The product was redistilled two times under a 0.01 to 0.001 mm. pressure at 130–140° before it was used.

*Preparation of Potassium Diacetone-d-Galacturonate from Diacetone-d-Galactose*—80 gm. of diacetone-d-galactose were oxidized with potassium permanganate under the exact conditions described by Ohle and Berend (2). The crude product was recrystallized from an alcohol-ether mixture and dried as stated above. The yield of the purified product was 70 gm., which is 70.8 per cent of that required for a theoretical yield.

*Rotation*— $[\alpha]_D^{20} = -66.0^\circ \pm 2.0^\circ$  (in water,  $c = 1.3$  per cent).

<i>Analysis</i> —Calculated for $C_{13}H_{17}O_7K \cdot \frac{1}{2}H_2O$ .	K 12.40
Found.	" 13.30

*Preparation of Potassium Diacetone-d-Galacturonate from d-Galacturonic Acid*—4.00 gm. of finely divided d-galacturonic acid were suspended in 500 cc. of dry acetone containing 2 cc. of concentrated sulfuric acid. The reaction mixture was shaken for 24 hours, whereupon approximately 75 per cent of the uronic acid dissolved. The unchanged d-galacturonic acid was filtered off and the filtrate exactly neutralized with alcoholic potassium hydroxide. After standing overnight the precipitate was filtered off and suspended in cold 95 per cent ethyl alcohol (15 cc.). The insoluble matter was filtered off and again suspended in 95 per cent ethyl alcohol (10 cc.). The precipitate remaining was collected on a filter and discarded. The filtrates from the first and second extractions were combined and added to 750 cc. of ether. After standing overnight the potassium diacetone-d-galacturonate crystallized out in fine needles. The yield was 2.0 gm. or 30.2 per cent of the theoretical yield.

*Rotation*— $[\alpha]_D^{20} = -65.0^\circ \pm 2.0^\circ$  (in water,  $c = 1.0$  per cent).

<i>Analysis</i> —Calculated for $C_{13}H_{17}O_7K \cdot \frac{1}{2}H_2O$ .	K 12.40
Found.	" 13.30

**Preparation of Diacetone-*d*-Galacturonic Acid from the Potassium Salt**—Following the conditions described by Ohle and Berend (2) 16.5 gm. of potassium diacetone-*d*-galacturonate were dissolved in 50 cc. of *N* sulfuric acid. The free acid was then extracted from the aqueous solution with a large quantity of ether. On drying the ethereal solution with sodium sulfate and subsequent evaporation of the solvent, 12.0 gm. of the crystalline acid were obtained. For analysis the compound was recrystallized from a mixture of benzene and petroleum ether. The yield of the crude product was 87.6 per cent of the theoretical.

**Melting Point**—The diacetone-*d*-galacturonic acid melted sharply at 157.5–158.0°.

**Rotation**— $[\alpha]_D^{20} = -84.0^\circ \pm 2.0^\circ$  (in chloroform,  $c = 1.07$  per cent).

**Analysis**—Calculated for  $C_{12}H_{18}O_7$ . N. E.\* 36.48 cc. 0.1 *N* alkali

Found. " 36.83 " 0.1 " "

\* N. E. represents the neutralization equivalent.

**Conversion of Diacetone-*d*-Galacturonic Acid to Barium *d*-Galacturonate**—9.4 gm. of crude diacetone-*d*-galacturonic acid were dissolved in 150 cc. of *N* sulfuric acid and allowed to stand at 40° for 36 hours. An excess of barium hydroxide was then added and after standing for a few minutes the solution was brought to neutrality by passing in carbon dioxide. The reaction mixture was heated on the steam bath for 30 minutes before the precipitated barium salts were filtered off. The filtrate was concentrated under diminished pressure to a small volume and after cooling was poured under stirring into 10 volumes of cold 95 per cent ethyl alcohol. The barium salt was isolated and purified by the procedure previously described in papers from this laboratory (4-7). By this procedure 7.5 gm. of barium *d*-galacturonate were obtained, or 83.7 per cent of the theoretical yield.

**Rotation**— $[\alpha]_D^{20} = +24.0^\circ \pm 1.0^\circ$  (in water,  $c = 1.3$  per cent).

**Analysis**—Calculated for  $(C_6H_8O_7)_2Ba$ . Ba 26.30

Found. " 25.70

**Conversion of Barium *d*-Galacturonate to *d*-Galacturonic Acid**—6.00 gm. of barium *d*-galacturonate (obtained from the diacetone acid) were dissolved in 15 cc. of water and to this solution 18.5

cc. of N sulfuric acid were added during the course of 15 minutes. 250 cc. of 95 per cent ethyl alcohol were then added and the reaction mixture placed in the ice chest for several days. The precipitate was filtered off and the filtrate concentrated under diminished pressure to 10 cc. 250 cc. of 95 per cent ethyl alcohol were added to the concentrate and the solution again placed in the ice chest for 2 days. The last traces of the precipitated barium *d*-galacturonate were removed and the clear solution concentrated *in vacuo* to 15 cc. Before the syrup could be removed from the flask spontaneous crystallization set in. The crystallized acid was dissolved in warm 90 per cent ethyl alcohol and after standing overnight in a desiccator containing calcium chloride the material crystallized in the form of a solid cake. After trituration with a small quantity of cold 95 per cent ethyl alcohol the acid was filtered off, washed with 95 per cent ethyl alcohol, and dried. By this procedure 3.0 gm. of crystalline *d*-galacturonic acid were obtained or 83.5 per cent of that required for a theoretical yield.<sup>1</sup>

**Melting Point**—The acid melted with decomposition at 157–159°.

**Rotation**— $[\alpha]_D^{20} = +51.0^\circ \pm 1.0^\circ$  (in water,  $c = 1.0$  per cent).

**Analysis**—Calculated for  $C_6H_{10}O_7 \cdot H_2O$ . N. E. 47.20 cc. 0.1 N alkali  
Found. " 47.30 " 0.1 " "

**Conversion of Potassium Diacetone-*d*-Galacturonate to  $\alpha$ -Methyl-*d*-Galacturonide Methyl Ester**—15.6 gm. of potassium diacetone-*d*-galacturonate were refluxed for 10 hours with 500 cc. of anhydrous methyl alcohol containing 2 per cent hydrogen chloride. The potassium chloride was then filtered off and the filtrate concentrated under diminished pressure to a small volume. After standing overnight the methylglycoside methyl ester that had crystallized out was filtered off and the filtrate taken up in methyl alcoholic hydrogen chloride and again methylated as above. This procedure was repeated for a third time, whereupon the products from the first, second, and third methylations were combined and twice recrystallized from 95 per cent ethyl alcohol. This last step is necessary to remove the potassium chloride which contaminates the crude product. The yield of crystalline  $\alpha$ -methyl-*d*-galactur-

<sup>1</sup> This represents the highest yield that we have obtained so far in this conversion.

onide methyl ester monohydrate was 6.5 gm. or 55.7 per cent of the amount required for a theoretical yield.

*Melting Point*—The compound melted at 137–138°.

*Rotation*— $[\alpha]_D^{20} = +121.0^\circ \pm 2.0^\circ$  (in water,  $c = 1.0$  per cent).

*Analysis*—Calculated for  $C_6H_{14}O_7 \cdot H_2O$ . OCH<sub>3</sub>, 24.90  
Found. " 24.40

*Preparation of  $\alpha$ -Methyl-d-Galacturonide from  $\alpha$ -Methyl-d-Galacturonide Methyl Ester*—3.70 gm. of  $\alpha$ -methyl-d-galacturonide methyl ester were dissolved in exactly 150 cc. of 0.2 N barium hydroxide and the reaction mixture allowed to stand at room temperature for 45 minutes. At the end of this period the barium hydroxide was exactly neutralized with 0.5 N sulfuric acid and the precipitated barium sulfate filtered off. The filtrate was concentrated just to dryness and then taken up in 150 cc. of warm 95 per cent ethyl alcohol. The insoluble matter was filtered away and the filtrate concentrated to 20 cc. The concentrate was placed in a crystallizing dish and after standing overnight over calcium chloride the crystalline  $\alpha$ -methyl-d-galacturonide which had separated was collected and dried in the usual manner. The yield was 2.7 gm. of the glycoside from 3.70 gm. of the ester glycoside, or 71.2 per cent of that required for a theoretical yield.

*Melting Point*—The acid melted sharply at 111.0–111.5° (8).

*Rotation*— $[\alpha]_D^{20} = +129.0^\circ \pm 1.0^\circ$  (in water,  $c = 1.3$  per cent).

*Analysis*

Calculated for  $C_7H_{12}O_7 \cdot 2H_2O$ . OCH<sub>3</sub>, 12.70, N. E. 41.00 cc. 0.1 N alkali  
Found. " 12.50, " 40.30 " 0.1 " "

*Preparation of  $\alpha$ -Methyl-d-Galacturonide from  $\alpha$ -Methyl-d-Galactoside*—The electrolytic oxidation apparatus of Isbell and Frush (12) was modified so that the reaction could be conducted at temperatures below 5°. The flask was replaced by an earthenware crock of 1 liter capacity, which was surrounded by a copper jacket so as to permit the circulation of cold water around the container. Additional cooling was accomplished with four immersion coolers placed in pairs adjacent to the electrodes. The electrodes were of graphite (diameter 25.4 mm.). The contents of the reaction chamber were violently agitated by a centrifugal stirrer throughout the entire reaction.

19.4 gm. of  $\alpha$ -methyl-*d*-galactoside, 25 gm. of calcium carbonate, 3 gm. of calcium bromide, and 750 cc. of water were charged into the above apparatus. The mixture was then electrolyzed at a potential of 12 volts and at a rate of 0.26 ampere per hour until the theoretical quantity of electricity (10.7 ampere hours) had passed through the solution. It is necessary to add 4 additional gm. of calcium bromide during the course of the reaction so as to replace any bromine that may be lost by volatilization.

The electrolyzed reaction mixture was removed from the cell and heated on the steam bath at 60° for 40 minutes. The insoluble matter was then removed and the filtrate concentrated under diminished pressure to a volume of 20 cc. The concentrate was filtered through an asbestos mat and the filtrate poured under stirring into 150 cc. of cold 95 per cent ethyl alcohol. After standing in the ice chest for several days the precipitate was centrifuged off and washed freely with 95 per cent ethyl alcohol until the washings were free of halogen. The precipitate was then dehydrated with absolute ethyl alcohol and anhydrous ether and finally dried over calcium chloride in a vacuum. By this procedure 7.0 gm. of calcium salt were obtained (calcium content 10.90 per cent).

6.5 gm. of the crude calcium salt were dissolved in 40 cc. of water and the requisite quantity of oxalic acid was added to remove the calcium from the solution. The calcium oxalate was collected on a filter and the filtrate concentrated under diminished pressure to a thick syrup. This syrup was taken up in 100 cc. of 95 per cent ethyl alcohol, filtered, and the filtrate again concentrated to a small volume. The syrup was transferred to a crystallizing dish and placed in a desiccator containing calcium chloride, whereupon the  $\alpha$ -methyl-*d*-galacturonide crystallized out after standing for several days. The crystalline acid was removed from the mother liquor and recrystallized from 95 per cent ethyl alcohol. The yield of the pure product was 0.75 gm., or 3.3 per cent of the theoretical yield.

*Melting Point*—The compound melted at 111.0–112.0° (8).

*Rotation*— $[\alpha]_D^{24} = +128.0^\circ \pm 1.0^\circ$  (in water,  $c = 1.0$  per cent).

*Analysis*

Calculated for  $C_7H_{12}O_7 \cdot 2H_2O$ . OCH<sub>3</sub>, 12.70, N. E. 41.00 cc. 0.1 N alkali  
Found. " 12.81, " 40.93 " 0.1 " "



## DISCUSSION

The original procedure of Ohle and Berend (2) for the preparation of diacetone-*d*-galacturonic acid was readily duplicated and the yields obtained were in close agreement with those reported. Ohle and Berend (2) first reported that diacetone-*d*-galacturonic acid has a melting point of 157°. Later Ohle and Dambergis (13) could not duplicate this value. Their preparation melted at 148°. We have confirmed the validity of the original value.

The diacetone-*d*-galacturonic acid prepared by the direct acetonation of *d*-galacturonic acid was found to be identical with the corresponding compound obtained by the oxidation of diacetone-*d*-galactose. Levene and Meyer (14) have shown that diacetone-*d*-galactose contains the pyranoid ring. Therefore the diacetone-*d*-galacturonic acid obtained directly from *d*-galacturonic acid most likely contains the same ring structure.

The oxidation of  $\alpha$ -methyl-*d*-galactoside to  $\alpha$ -methyl-*d*-galacturonide was suggested by the investigations of Bergmann and Wolff (15) and Smolenski (16). The former reported that  $\alpha$ -menthol-*d*-glucoside is oxidized to  $\alpha$ -menthol-*d*-glucuronide with alkaline bromine but the yield obtained was very poor (17). An attempt to oxidize  $\alpha$ -methyl-*d*-glucoside to  $\alpha$ -methyl-*d*-glucuronide led only to the isolation of glyoxylic acid. Smolenski (16) reported that  $\alpha$ -methyl-*d*-glucuronide was formed by the oxidation of  $\alpha$ -methyl-*d*-glucoside with alkaline bromine, or with hydrogen peroxide, in yields up to 30 per cent of the theoretical amount. These claims, however, are based on very meager evidence and an attempt to duplicate this reaction was unsuccessful (unpublished data obtained by our colleague, Mr. Sam Morell (18)).

The method used by Isbell and Frush for the preparation of aldonic acids seemed to offer ideal conditions for an attempt to oxidize  $\alpha$ -methyl-*d*-galactoside to  $\alpha$ -methyl-*d*-galacturonide. However, even under these apparently mild conditions only a 3 per cent yield of  $\alpha$ -methyl-*d*-galacturonide was obtained.<sup>2</sup> While the low yield prohibits the use of this method for the preparation of

<sup>2</sup> The oxidation of  $\alpha$ -methyl-*d*-galactoside with alkaline potassium or barium permanganate did not yield a detectable amount of  $\alpha$ -methyl-*d*-galacturonide (unpublished results of the authors).

*d*-galacturonic acid, the reaction itself offers evidence supporting the pyranoside structure of  $\alpha$ -methyl-*d*-galacturonide previously prepared by the methylation of *d*-galacturonic acid<sup>3</sup> (8-10). Since the structure of  $\alpha$ -methyl-*d*-galactopyranoside has been proved by Haworth and coworkers (20), the oxidation of this compound should yield  $\alpha$ -methyl-*d*-galacturonopyranoside. The rotation of the glycuronide prepared by the oxidation of the glycoside was identical with that of the glycuronide prepared from *d*-galacturonic acid, thereby proving their structural identity.

#### SUMMARY

Crystalline *d*-galacturonic acid has been prepared through the oxidation of diacetone-*d*-galactose with potassium permanganate and subsequent hydrolysis of the diacetone-*d*-galacturonic acid to the free hexuronic acid. The yield of crystalline *d*-galacturonic acid from crystalline *d*-galactose was 30 per cent of the theoretical amount.

Diacetone-*d*-galacturonic acid has been prepared by the direct acetonation of *d*-galacturonic acid and was found to be identical with the acid prepared by the oxidation of diacetone-*d*-galactose.

$\alpha$ -Methyl-*d*-galactoside has been oxidized to  $\alpha$ -methyl-*d*-galacturonide and the structural implications deducible from this reaction have been pointed out.

We are indebted to our colleague, Dr. Eugene Schoeffel, for conducting the microanalytical determinations.

*Addendum*—Since the completion of this investigation Haworth and Hirst (21) and Reichstein *et al.* (22) have reported the preparation of *d*-galacturonic acid in their synthetic studies on ascorbic acid by methods similar to those described above.

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## THE COMPOSITION OF AN ALDOBIONIC ACID FROM FLAXSEED MUCILAGE\*

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In 1930, Anderson and Crowder (1) reported that flaxseed mucilage yields on hydrolysis, an aldobionic acid composed of *l*-rhamnose and *d*-galacturonic acid. The free galacturonic acid was not isolated in the crystalline form, nor as a crystalline derivative. The presence of *d*-galacturonic acid was inferred from oxidation studies. Mucic acid was obtained when the aldobionic acid was oxidized with bromine and hydrobromic acid after the method of Heidelberger and Goebel (2). Whereas this procedure established the presence of the galactose configuration, it does not reveal which isomer of galacturonic acid is present; *e.g.*, *d*-, *l*-, or *dl*-. The asymmetric center of the galactose configuration possesses a plane of symmetry; consequently the same dicarboxylic acid, *e.g.* mucic acid (inactive), is obtained by oxidation from all of the stereoisomeric forms of galactose or the corresponding aldehyde sugar acids (3).

Since Anderson has recently reported the occurrence of *l*-galactose in flaxseed mucilage (4) it becomes desirable to know which stereoisomeric form of galacturonic acid is present in the aldobionic acid. To date only the *d* form of galacturonic acid has been found in nature (5, 6). The occurrence of the rather rare *l* form of galactose in the mucilage from the flaxseed raises the possibility that the corresponding *l* form of galacturonic acid might be present in the aldobionic acid. The question was answered by the following direct method.

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## EXPERIMENTAL

The barium aldobionate prepared after the directions of Anderson and Crowder (1) was hydrolyzed for 16 hours at 100° with 2.5 per cent sulfuric acid. From the hydrolysate the uronic acid was isolated as its barium salt in the usual manner (7) and finally as the free acid, m.p. 157–158°,  $[\alpha]_D^{20} = +54.0^\circ$ . The brucine salt melted at 188–189° and showed  $[\alpha]_D^{28} = -7.5^\circ$ . These values are in excellent agreement with the accepted constants for *d*-galacturonic acid and its brucine salt (8). The above data therefore establish conclusively that the *d* form of galacturonic acid is present in the aldobionic acid originally described by Anderson and Crowder (1). Incidentally in the course of this work we were able to confirm the occurrence of *l*-rhamnose, m.p. 94–95°,  $[\alpha]_D^{20} = +8.0^\circ$ , reported by the same authors, as the sugar component of the aldobionic acid.

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# THE EFFECT OF INSULIN AND EPINEPHRINE ON THE AMINO ACID CONTENT OF THE BLOOD OF ADRENALECTOMIZED RABBITS

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The administration of insulin to normal animals has been shown by Luck and his collaborators (1-3) to cause hypoaminoacidemia. The observations have been confirmed by Kerr and Krikorian (4) and Powers and Reis (5).

In the course of an inquiry into the rôle of hypoglycemia in the production of the amino acid lowering, Luck and Morse (6) observed that epinephrine, as effectively as insulin, would cause hypoaminoacidemia. This and other observations reported by Davis, Luck, and Miller (7) on the behavior of partially inactivated insulin suggested to us the possibility that epinephrine rather than insulin may be the responsible agent in so called insulin hypoaminoacidemia. On the assumption that administration of insulin would induce an increased secretion of epinephrine, a possibility, indeed, which seems proved by many investigations (8), it would be possible so to explain the established facts pertaining to this phenomenon.

The purpose of the present paper is to report the results of experiments designed to test the validity of this hypothesis.

## EXPERIMENTAL

*Operative Procedure*—The adrenals of rabbits weighing from 2 to 3 kilos were freed from the surrounding tissue and an electric cautery applied to the gland in such a manner that the medullary portion was completely destroyed while the cortical destruction was kept at a minimum. This procedure was found to prolong the life of the animals greatly, and at the same time to have an

effect similar to complete adrenalectomy without entire loss of cortical tissue. It is exceedingly unlikely that the adrenal medullæ were functional because of the positive nature of the results here reported and also because there was no transient hyperglycemia following severe fright. Prior to their use in these experiments, the animals were permitted at least 1 week in which to recover from the operation.

*Analytical Methods*—Danielson (9) has recently published a modification of the Folin (10) colorimetric method for the determination of amino acid nitrogen. This method has many advantages over the original Folin method and for this reason it was used in this work. It was first checked, however, by using both methods in duplicate determinations in several of the experi-

TABLE I  
*Effect of Insulin on Normal Rabbits*

The figures for blood content analyses are measured in per cent; 100 per cent represents the value at 0 hours in each instance.

No. of rabbits used	Dose	Amino acid N					Inorganic P					Reducing sugar							
		1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.	1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.	½ hr.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	5 hrs.	8 hrs.	
	unit per kg.																		
6	0.25	97	90	88	85	93	81	77	77	85	96	72	55	53	51	56	71	92	
6	1	98	92	85	80	80	80	66	62	66	74	75	49	39	40	40	43	44	

ments. The results agreed qualitatively, although the results by the Danielson method were slightly lower than those obtained by the Folin method.

Since both insulin and epinephrine alter the inorganic phosphorus and reducing sugar content of blood, it was thought wise to analyze the blood for these constituents as well as for amino acid nitrogen. The determinations of reducing sugar and inorganic phosphorus were made by the methods of Folin (11) and Fiske and Subbarow (12) respectively.

*Experiments with Insulin on Normal Rabbits*—To test the potency of the insulin used (Lilly's U-100), doses of 0.25 unit and 1 unit per kilo of body weight were injected subcutaneously into a number of normal fasting rabbits. Six rabbits were used for each

TABLE II

*Effect of 0.25 Unit of Insulin per Kilo on Adrenalectomized Rabbits*

The figures for blood content analyses are measured in per cent; 100 per cent represents the value at 0 hours in each instance.

Rabbit No.	Elapsed time between operation and experiment	Weight	Amino acid N				
			1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.
	days	kg.					
P-63	7	2.6	99	98	90	95	104
290	9	2.6	105	*			
291	9	2.8	105	105	100	98	94
292	9	2.0	94	*			
293	9	2.0	102	105	101	98	97
P-63	14	2.4	98	103	101	98*	92
P-65	14	2.0	97	96	98*	91	82
291	16	2.9	99	107	100	112*	97
293	16	2.0	106	97	104*	104	95
294	15	2.8	95	101	103*	107	93
W-310	11	2.3	100	103	103	102*	93
Mean values.....			100	101	100	100	94

Rabbit No.	Inorganic P					Reducing sugar					
	1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.	1 hr.	1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.
P-63	97	89	84	99	101	89	59	66	60	94	104
290	90	*				76	46	*			
291	84	77	78	79	74		58	47	46	51	92
292	87	*				70	42	*			
293	85	84	79	81	79	78	52	45	49	70	110
P-63	97	95	95	100*	88	77	71	55	40	*	
P-65	87	86	74*	77	91	91	64	50	*		
291	80	82	77	78*	82	86	53	33	47	*	
293	84	82	73*	76	68	81	57	36	*		
294	85	78	74*	72	71	78	56	34	*		
W-310	95	84	86	81*	94	84	59	52	44	*	
Mean values..	87	82	78	80	82	81	56	46	46	67	102

\* Convulsions occurred prior to the taking of this sample. 20 cc. of 10 per cent glucose solution were injected subcutaneously.

dose. The results of these experiments are summarized in Table I. The expected lowering of amino acid nitrogen content was observed.



*Experiments with Insulin on Adrenalectomized Rabbits*—Tables II and III show the results obtained following the administration of 0.25 and 1 unit of insulin per kilo of body weight to fasting adrenalectomized rabbits. 0.25 unit of insulin per kilo was found to be sufficient to reduce the blood sugar content to the convulsive level in the adrenalectomized rabbits. Luck and Morse (6) have shown that when the sugar content of the blood is maintained at the preinjection level or above, insulin will still produce a hypo-

TABLE III

*Effect of 1 Unit of Insulin per Kilo on Adrenalectomized Rabbits\**

The figures for blood content analyses are measured in per cent; 100 per cent represents the value at 0 hours in each instance.

Rabbit No.	Elapsed time between operation and experiment	Weight	Amino acid N				
			1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.
	<i>days</i>	<i>kg.</i>					
P-63	34	2.3	102	100	103	94	100
291†	36	2.7	100	97	100	102	101
293	36	2.3	105	109	105	104	101
294	35	3.1	101	98	101	100	93
298	25	2.0	99	102	97	100‡	93
W-310	31	2.3	99	100	100	100	
Mean values . . . . .			101	101	101	100	98

\* 20 cc. of 10 per cent glucose were administered hourly to each rabbit during the course of the experiment to prevent convulsions.

† By mistake this rabbit received a total of 3.7 units of insulin instead of 2.7 units.

‡ This rabbit was in convulsions during the taking of this sample.

aminoacidemia. For this reason, 20 cc. of a 10 per cent glucose solution were injected into the rabbits when convulsions occurred and the withdrawal of samples for amino acid nitrogen and inorganic phosphorus analyses was continued throughout the remainder of the experiment. In the experiment in which 1 unit of insulin per kilo was administered to the adrenalectomized animals, convulsions were prevented by the repeated administration of 10 per cent glucose solution. In one case, however, convulsions ensued even though the animal had received more than

40 cc. of 10 per cent glucose subcutaneously over a period of 5 hours prior to the convulsive seizure.

It will be seen from these experiments that insulin caused no lowering of the amino acid nitrogen content of the blood of adrenalectomized rabbits. In fact, in many cases the amino acid nitrogen content was above the preinjection level, amounting to an average of 11 per cent in one experiment to be mentioned later. We consider that this hyperaminoacidemia following insulin injection may be significant. The group of rabbits to which 0.25 unit of insulin per kilo was administered (Table II) had not previously been used in insulin work and was not sensitive to the hormone. In later experiments, however, they showed a marked hyperaminoacidemia following small doses of insulin (0.25 unit per kilo) although a large dose (1 unit per kilo) elicited no such rise in blood amino acid content. It is possible that insulin in small amounts may produce a transient hyperaminoacidemia.

*Experiments with Insulin and Epinephrine on Adrenalectomized Rabbits*—In order to demonstrate the difference in the action of insulin and epinephrine, the following experiment was devised: Into six adrenalectomized rabbits 0.25 unit of insulin per kilo of body weight was injected subcutaneously. 5 cc. blood samples were withdrawn from a marginal ear vein before the injection and 1 and 2 hours after the injection. In addition, 0.1 cc. blood samples were withdrawn for reducing sugar determinations at the  $\frac{1}{2}$  and  $1\frac{1}{2}$  hour periods. Immediately after the 2 hour sample had been taken, 0.2 mg. of epinephrine (Armour's supranaline) per kilo was injected. Blood samples were then taken at the 3, 4, 5, 7, and 10 hour periods (after insulin injection). In addition, blood sugar samples were taken at the  $2\frac{1}{2}$  and  $3\frac{1}{2}$  hour periods. The results of these experiments are given in Table IV.

In this experiment the amino acid nitrogen content of the blood rose to a point higher than the preinjection level following the administration of insulin. This has already been commented upon. Following the injection of epinephrine a marked drop in the amino acid nitrogen content is noted.

The inorganic phosphorus content of the blood shows a drop both with insulin and epinephrine. This observation confirms the work of Ellsworth and Weinstein (13) who concluded from their work on adrenalectomized dogs that both insulin and epinephrine caused a hypophosphatemia independently.

The blood sugar curve obtained in this experiment is of interest. The drop in blood sugar content in the first 2 hours following the insulin injection closely approximates that obtained by injecting

TABLE IV

*Effect of Insulin\* and Epinephrine upon Adrenalectomized Rabbits*

The figures for blood content analyses are measured in per cent; 100 per cent represents the value at 0 hours in each instance.

Rabbit No.	Elapsed time between operation and experiment	Weight	Amino acid N								Inorganic P							
			1 hr.	2 hrs.†	3 hrs.	4 hrs.	5 hrs.	7 hrs.	10 hrs.	1 hr.	2 hrs.†	3 hrs.	4 hrs.	5 hrs.	7 hrs.	10 hrs.		
	days	kg.																
P-63	28	2.5	115	106	92	77	76	78	83	88	79	65	60	69	64	60		
291	30	2.8	112	102	94	75	86		100	86	84	67	65	69		67		
293	30	2.3	110	107	87		94	83	93	96	92			71	68	70		
294	29	3.1	117	102	87†	82	86	93	93	98	94	89†	85	86	72	68		
298	19	2.1	112	105†	79	83	80	88	77	99	†	75	72	73	67	70		
W-310	25	2.6	103	101	83			85	92	96		74			87	82		
Mean values.....			111	104	87	79	84	85	89	93	87	74	70	73	71	69		

Rabbit No.	Reducing sugar										
	½ hr.	1 hr.	1½ hrs.	2 hrs.†	2½ hrs.	3 hrs.	3½ hrs.	4 hrs.	5 hrs.	7 hrs.	10 hrs.
P-63	85	58	53	51	77	104	106	145	172	126	105
291	96	55	49	44	88	123	135	154	170	150	109
293	74	54	50	41	90	169	170	155	181	160	113
294	72	45	30	21	†						
298	51	34	†								
W-310	63	40	38	37	80	190	184	208	189	145	109
Mean values.		77	47	42	39	84	146	149	165	178	109

\* 0.25 unit of insulin per kilo was injected immediately after the sample was taken at 0 hours.

† 0.2 mg. of epinephrine per kilo was injected immediately after this sample was taken.

‡ Convulsions occurred prior to the taking of this sample; 20 cc. of 10 per cent glucose were injected.

1 unit of insulin per kilo into normal animals. A very marked and rapid rise in blood sugar level is observed after the injection of epinephrine. It is interesting to note from a plot of the results

that when the blood sugar concentration reached a value of about 145–150 mg. per cent, the curve flattened and this was followed by another rise to the maximum of about 175 mg. per cent. This was observed in three out of four cases (Table IV). The significance of this observation is doubtful, and sufficient data have not been accumulated with which to explain the phenomenon satisfactorily.

*Experiments with Epinephrine on Adrenalectomized Rabbits*—Luck and Morse (6) have shown that epinephrine lowers the amino acid nitrogen content of the blood in normal animals. No complete study seems to have been made upon the effect of epinephrine on the amino acid content of the blood of adrenalectomized animals. In the experiments about to be described two different doses of epinephrine were used: 0.1 mg. per kilo and 0.2 mg. per

TABLE V  
*Effect of Epinephrine on Adrenalectomized Rabbits*

The figures for blood content analyses are measured in per cent; 100 per cent represents the value at 0 hours in each instance.

No of rabbits used	Dose	Amino acid N					Inorganic P					Reducing sugar					
		1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.	1 hr.	2 hrs.	3 hrs.	5 hrs.	8 hrs.	1 hr.	1½ hrs.	2 hrs.	3 hrs.	5 hrs.	8 hrs.
	mg. per kg																
6	0.1	97	93	87	84	82	88	80	80	81	83	117	151		151	143	120
5	0.2	91	80	81	77	76	81	77	89	96	94	167	233	262	259	192	143

kilo. Six rabbits were used in each experiment and blood samples were taken at the 0, 1, 2, 3, 5, and 8 hour periods. The results of these experiments are summarized in Table V. Reference to Table V shows that epinephrine produces a marked reduction in the amino acid nitrogen and inorganic phosphorus contents of the blood.

*Control Experiments*—Luck and Morse (6) and Davis, Luck, and Miller (7) report that prolonged hemorrhage produces a hypoaminoacidemia. For this reason, control experiments were performed in which physiological saline was injected into six rabbits and 5 cc. blood samples were withdrawn at the 0, 1, 2, 3, 5, and 8 hour periods. A reduction in the amino acid nitrogen content of the blood of about 4 per cent was noted. This is not great

enough to affect the validity of the results in the previous experiments. The other blood constituents remained constant.

Since epinephrine altered the amino acid content of the blood of the adrenalectomized animals in a comparable manner to that observed in normal animals and since a considerable period of time elapsed between the operations and most of the experiments, it was not deemed necessary to perform control operations in order to ascertain whether the observed results were affected by trauma.

#### DISCUSSION

These experiments clearly demonstrate that insulin does not cause a hypoaminoacidemia in adrenalectomized rabbits. Since insulin produces a lowering of amino acid nitrogen content in the blood of normal rabbits and epinephrine produces a similar effect in both normal and adrenalectomized rabbits, it is concluded that the observed lowering of amino acid nitrogen in the blood of normal animals following an insulin injection is due not to the insulin but to the increased secretion of epinephrine elicited by the effects of the insulin injection. Hence, it is concluded that insulin has no direct action on the amino acid content of the blood and tissues and that all the observations to this effect reported in the literature can be ascribed to the resultant increased secretion of epinephrine.

The ability of insulin, acting indirectly through the adrenals, to cause hypoaminoacidemia occurs to us as a possible means of testing for the adequacy of medullary function. Experiments to this end are contemplated.

#### SUMMARY

1. A study of the effect of insulin and epinephrine upon the amino acid nitrogen, inorganic phosphorus, and reducing sugar content of the blood of adrenalectomized rabbits has been made.

2. It has been shown that insulin does not cause a hypoaminoacidemia in adrenalectomized rabbits.

3. It has been shown that epinephrine produces a hypoaminoacidemia in adrenalectomized rabbits.

4. It is concluded that the observed hypoaminoacidemia produced in normal animals following an insulin injection is due entirely to an increased secretion of epinephrine by the animal itself.

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## **FACTORS IN FOOD INFLUENCING HEMOGLOBIN REGENERATION**

### **III. EGGS IN COMPARISON WITH WHOLE WHEAT, PREPARED BRAN, OATMEAL, BEEF LIVER, AND BEEF MUSCLE**

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In former papers (1, 2) it has been shown that the iron of wheat is very efficiently used in the regeneration of hemoglobin in rats made anemic by milk feeding, whether fed as ground whole wheat or as bran prepared for human consumption by steaming and cutting into rather fine particles. In a 6 week period for hemoglobin regeneration, the best results have been obtained with 6 gm. of whole wheat furnishing 0.19 mg. of iron per rat per day, or with bran enough to supply the same amount of iron. Similar results have been secured with 6 gm. of oatmeal containing the same amount of iron and slightly more copper than the whole wheat.

Since eggs, liver, and beef muscle are all important sources of iron in the dietary, it seemed desirable to make a study of these foods in comparison with these cereal products; and the results form the subject of the present paper. By feeding whole wheat at different levels (1) it was found that the increases in hemoglobin were quite closely proportional to the amount of the food fed, and in these subsequent studies the curves of regeneration with 1.5, 3, and 6 gm. respectively of whole wheat per day have been compared with those from these other foods and from combinations with bran and whole wheat furnishing equivalent amounts of iron.

During the time which has elapsed since these investigations were begun, the relationship of both iron and copper to hemoglobin regeneration has received much attention, and it now seems well established that iron absorption and storage may take place inde-



pendently of copper, but that the utilization of iron in hemoglobin formation is definitely influenced by copper. Rose and Vahlteich (1) found that supplementing 3 gm. of white flour with ferric chloride to make it equal in iron to 6 gm. of whole wheat or with copper sulfate to make it equal in copper caused very little increase in hemoglobin, but that when both were added the increase was much greater, though not quite equal to that due to whole wheat. Cunningham (3) in 1931 showed that iron was stored in the liver in absence of copper, but that such iron was not used for hemoglobin production till copper was also provided, and Cook and Spilles (4) observed a similar effect of copper upon the utilization of iron stored in the spleen. Josephs (5) has also reported that copper has no influence on iron retention but does determine the proportion of the retained iron found in the liver. These studies have been further extended by Elvehjem and Sherman (6) who find that when pure iron is fed to anemic rats for a period of 2 weeks there is a decided increase in the total iron content of the liver and spleen, but no increase in the amount of hemoglobin in the blood. Subsequent feeding of copper caused a temporary rise in hemoglobin accompanied by a depletion of the store in the liver.

#### EXPERIMENTAL

In testing foods for their hemoglobin-regenerating power, it is important that reserves of both iron and copper be depleted as uniformly as possible in all the test animals. In our work the same grade of mixed certified milk has been used throughout and the animals have been depleted to as nearly uniform a state of anemia as possible, but not so low as to keep the animals long in an anemic state. This would make recovery unduly difficult and more irregular. They have reached the desired level (4 to 6 gm. of hemoglobin per 100 cc. of blood) at ages sufficiently close together (62 to 79 days) to rule out the factor of age itself as a cause of variation in total body iron. The uniformity of the iron content of the bodies of these anemic rats is shown by the work of Rose and Kung (2), who found that 78 rats reduced to approximately 6 gm. of hemoglobin per 100 cc. of blood averaged  $0.0021 \pm 0.00003$  per cent of iron.

The details of technique described by Rose and Vahlteich (1) have been followed in this series of investigations. Anemic rats

kept on a milk diet have been fed a supplement whose iron and copper content was known, and their hemoglobin has been tested at suitable times by the Newcomer method.

In the study of the egg, dried whole egg was first used, in amount to yield about the same iron as 3 gm. of whole wheat, and as 3 gm. of beef muscle. The differences among these three foods were surprisingly large. With the 3 gm. of beef muscle there was no

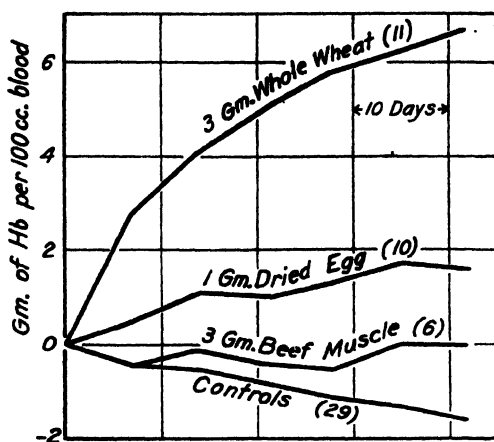


FIG. 1. Curves of hemoglobin regeneration on dried whole egg and fresh lean beef to furnish approximately the same amount of iron as 3 gm. of whole wheat. The figures in parentheses indicate the number of animals.

	In supplement		Estimated total intake	
	Fe	Cu	Fe	Cu
	mg.	mg.	mg.	mg.
1 gm. dried whole egg....	0.083	0.007	0.10-0.12	0.014-0.020
3 " fresh beef muscle. ....	0.090	0.007	0.11-0.12	0.015-0.018
3 " whole wheat. ....	0.10	0.023	0.12-0.14	0.031-0.037

increase whatever in hemoglobin, and the average increase from feeding the egg was only 1.5 gm. for the 6 weeks, while that for the 3 gm. of whole wheat was 6.7 gm. These differences, shown in Fig. 1, may now be related in part, at least, to the relative amounts of copper, the quantity of this element furnished by the whole wheat being over 3 times that furnished by the egg or beef muscle.

In further studies, dried egg yolk was substituted for the dried whole egg, and an amount of this to furnish about the same quantity of iron as 3 gm. of whole wheat was also compared with dried liver and with dried and cooked fresh spinach, each yielding a

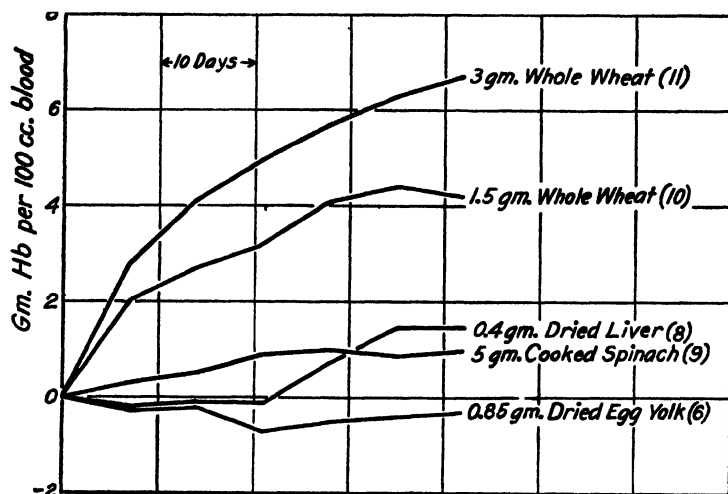


FIG. 2. Curves of hemoglobin regeneration on dried egg yolk, liver, and cooked spinach in comparison with 1.5 and 3 gm. of whole wheat. The figures in parentheses indicate the number of animals.

	In supplement		Estimated total intake	
	Fe	Cu	Fe	Cu
	mg.	mg.	mg.	mg.
0.85 gm. dried egg yolk.....	0.10	0.0035	0.12-0.14	0.009-0.017
0.4 " " liver.....	0.10	0.025	0.13-0.14	0.036-0.039
5 " cooked spinach (0.4 gm. dry weight).....	0.10	*	0.13-0.14	
1.5 gm. whole wheat.....	0.048	0.012	0.08	0.020-0.023
3 " " ".....	0.10	0.023	0.12-0.14	0.031-0.037

\* According to Mitchell and Miller, 0.0028 mg.; according to Levine, 0.0015 mg.

similar amount of iron. Here again, there was practically no increase with any of these foods except the wheat, as shown in Fig. 2. In the case of the liver, copper is not the limiting factor, as its content is as high as that of 3 gm. of whole wheat. This is

further substantiated by a comparison of liver with whole wheat flour furnishing the same amount of iron. Again the liver, higher in copper, caused less regeneration than the whole wheat, as shown in Fig. 3.

Rose and Kung (2) found that it was necessary to use twice as much iron in the form of liver as of whole wheat to get a given increase in hemoglobin in 6 weeks. Elvehjem (7) has shown that hematin is less readily utilized than inorganic iron, and this would

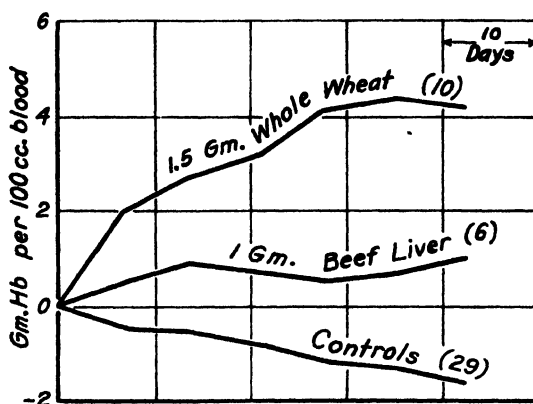


FIG. 3. Curves of hemoglobin regeneration on 1 gm. of fresh beef liver in comparison with 1.5 gm. of whole wheat flour. The figures in parentheses indicate the number of animals.

	In supplement		Estimated total intake	
	Fe	Cu	Fe	Cu
	mg.	mg.	mg.	mg.
1 gm. fresh beef liver.....	0.043	0.019	0.07-0.08	0.028-0.032
1.5 " whole wheat.....	0.048	0.012	0.08	0.020-0.023

seem to be the explanation of the difference between liver and whole wheat. But in the case of the egg the limiting factor is not the organic form of the iron, but the relatively low content of copper, since practically the same regeneration was secured with the egg yolk and its ash. To test the influence of copper, 1.7 gm. of dried egg yolk or its ash were supplemented by iron, copper, and iron plus copper. Addition of iron caused only a slight increase over the amount of regeneration with egg yolk alone, while

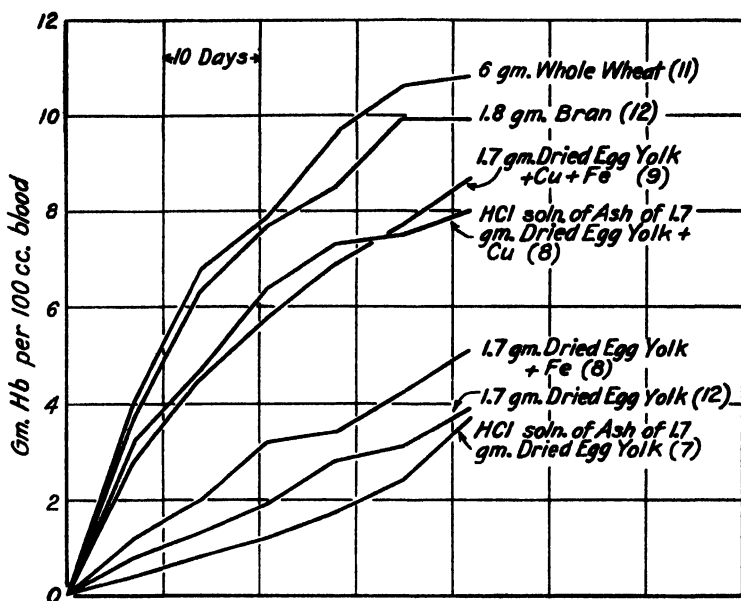


FIG. 4. Curves of hemoglobin regeneration on dried egg yolk and its ash, alone or supplemented with iron, copper, or iron plus copper, in comparison with 6 gm. of whole wheat and 1.8 gm. of prepared bran. The figures in parentheses indicate the number of animals.

	In supplement		Estimated total intake	
	Fe	Cu	Fe	Cu
	mg.	mg.	mg.	mg.
1.7 gm. dried egg yolk.....	0.20	0.007	0.22-0.27	0.013-0.022
Ash of 1.7 gm. dried egg yolk ...	0.20	0.007	0.23-0.24	0.016-0.019
1.7 gm. dried egg yolk and FeCl <sub>3</sub> ..	0.30	0.007	0.31-0.34	0.011-0.019
1.7 " " " " FeCl <sub>3</sub> and CuSO <sub>4</sub> .....	0.30	0.043	0.32-0.34	0.050-0.057
Ash of 1.7 gm. dried egg yolk and CuSO <sub>4</sub> .....	0.20	0.043	0.23-0.26	0.053-0.063
1.8 gm. prepared bran.....	0.20	0.018	0.23-0.24	0.029-0.035
6 " whole wheat.....	0.19	0.043	0.21-0.24	0.049-0.059

addition of copper brought a response nearly but not quite equivalent to that with bran furnishing the same amount of iron, as shown in Fig. 4.

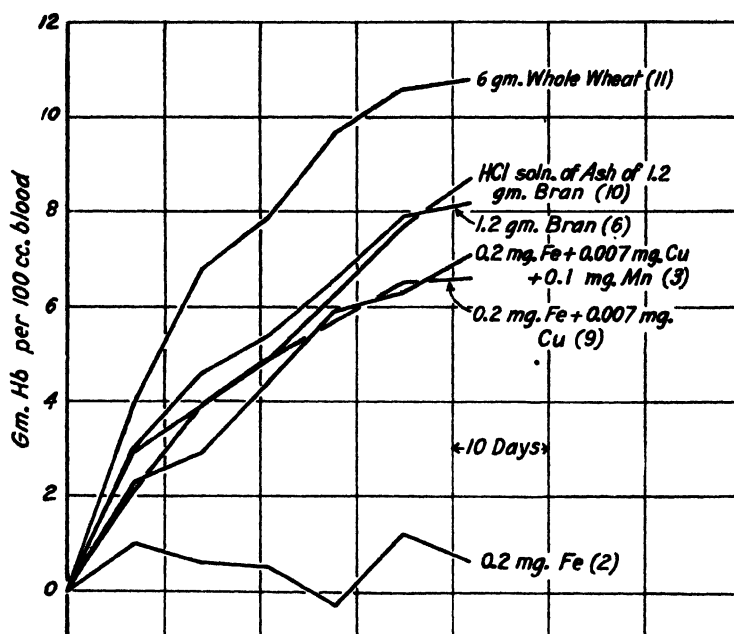


FIG. 5. Curves of hemoglobin regeneration on 6 gm. of whole wheat; on prepared bran, and its ash; on ferric chloride and copper sulfate to equal 1.7 gm. of dried egg yolk; on these mineral supplements with the addition of 0.1 mg. of manganese; and on ferric chloride as the only addition to the milk diet. The figures in parentheses indicate the number of animals.

	In supplement		Estimated total intake	
	Fe	Cu	Fe	Cu
	mg.	mg.	mg.	mg.
6 gm. whole wheat .....	0.19	0.043	0.21-0.24	0.049-0.059
1.2 " prepared bran .....	0.20	0.012	0.23-0.24	0.021-0.027
Ash of 1.2 gm. prepared bran ...	0.20	0.012	0.24-0.25	0.024-0.029
FeCl <sub>3</sub> and CuSO <sub>4</sub> .....	0.20	0.007	0.22-0.25	0.014-0.027
" CuSO <sub>4</sub> , and 0.1 mg. Mn ...	0.20	0.007	0.24-0.25	0.020-0.023
" .....	0.20	0.000	0.22-0.23	0.007-0.011

But these results did not yet equal those with whole wheat; hence some further comparisons were made of (1) bran, (2) bran ash, (3) iron and copper equal to the amounts of each metal in 1.7 gm. of dried egg yolk, (4) same as (3) plus 0.1 mg. of manganese.

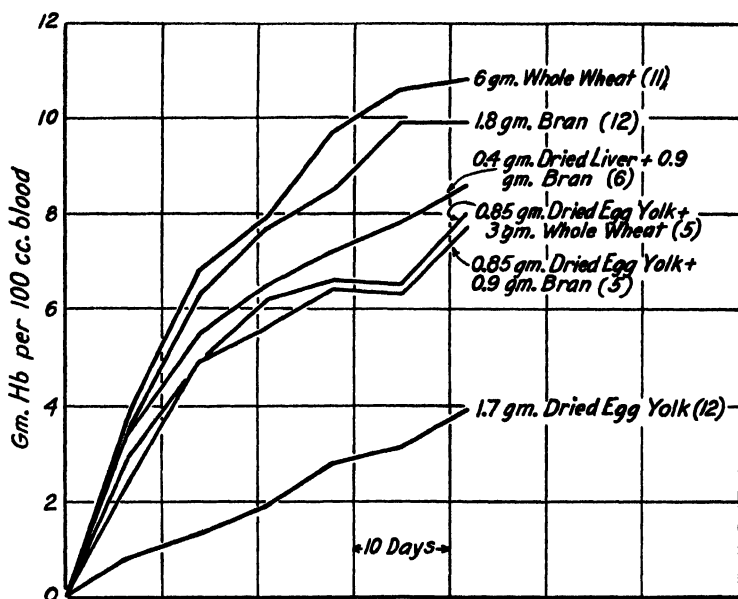


FIG. 6. Curves of hemoglobin regeneration on combinations of liver and bran, egg yolk and whole wheat, egg yolk and bran in comparison with egg yolk, bran, and whole wheat separately. The figures in parentheses indicate the number of animals.

	In supplement		Estimated total intake	
	Fe	Cu	Fe	Cu
	mg.	mg.	mg.	mg.
0.4 gm. dried liver and 0.9 gm. prepared bran .....	0.20	0.034	0.24-0.25	0.047-0.050
0.85 gm. dried egg yolk and 3.0 gm. whole wheat .....	0.20	0.024	0.22-0.27	0.032-0.040
0.85 gm. dried egg yolk and 0.9 gm. prepared bran .....	0.20	0.012	0.24-0.28	0.024-0.030
1.7 gm. dried egg yolk .....	0.20	0.007	0.22-0.24	0.013-0.022
1.8 " prepared bran .....	0.20	0.018	0.23-0.25	0.029-0.035
6 " whole wheat .....	0.19	0.043	0.21-0.24	0.049-0.059

The results with bran and bran ash were practically the same (8.2 and 8.7 gm. of hemoglobin per 100 cc. of blood), and again the pure iron and copper salts equivalent in amount to these elements in

the egg yolk gave somewhat lower results (6.8 gm.). Addition of manganese was without effect in the case of the three animals tested. None of these preparations proved quite equal to whole wheat, containing the same amount of iron but a higher proportion of copper (Fig. 5), and so some further tests were made of the influence of whole wheat or bran in combination with egg yolk and with liver in such proportions that each food in the combination yielded 50 per cent of the total iron. In these combinations the total iron was the same (0.2 mg.) but the copper varied from 0.012 to 0.034 mg. The results differed little, the hemoglobin increases ranging from 7.8 to 8.6 gm. per 100 cc. and approximating closely the value for egg yolk supplemented by copper sulfate yielding the same amount of iron and 0.043 mg. of copper (Figs. 4 and 6). It would seem that 0.2 mg. of iron and 0.012 mg. of copper furnish very good but not quite the optimum proportions of these elements for maximum hemoglobin rebuilding, since better results were obtained with 0.2 mg. of iron and 0.018 mg. of copper in the form of 1.8 gm. of bran.

The chief limiting factor in egg yolk appears therefore to be copper. The results with liver in combination with bran are not as good as would be expected from the liberal amount of copper and here the less available iron from the liver is doubtless the limiting factor. It will be noted in Fig. 6 that none of the combinations is quite as efficient as whole wheat or bran, and it still seems that there is some other factor in wheat which makes it specially efficient for hemoglobin regeneration. The same is true of oatmeal. These three foods have given consistently higher values than others with equal amounts of iron and copper. One factor to take into consideration is the total intake of iron and copper. When the ash is used, more milk is consumed by the animal to obtain the other nutrients required. We have in each case estimated from analyses of the milk and the records of food consumed the iron and copper derived from the milk and have found that while the amount of iron from the milk was often not more than 10 or 15 per cent of the total iron, the copper from milk furnished in some cases more than the supplement, as it ranged from 0.007 to 0.02 mg. per day. If the total intake is taken into account, the best regeneration occurred with approximately 0.26 mg. of iron and 0.05 to 0.06 mg. of copper daily.



## DISCUSSION

It is difficult to compare our results with the few data available from the work of other investigators. So far as we know, there are no studies on eggs. In many instances the iron or copper or both have been fed in amounts considerably above those required to achieve a hemoglobin increase of 10 to 11 gm. in 6 weeks and in other studies the number of animals has been too small to give reliable results. In some investigations copper has not been reported and in other cases, differences in the diet fed the mother and shared by the young in the latter part of the lactation period might cause differences in the relative amounts of iron or copper stored by the young. The amount of copper in the milk fed has undoubtedly varied as already pointed out by one of us (8), reported analyses of copper in milk ranging from 0.12 to 0.8 mg. per liter. Waddell, Elvehjem, Steenbock, and Hart (9) fed 0.5 gm. of dried beef liver furnishing 0.15 mg. of iron six times per week and obtained an increase of about 5 gm. of hemoglobin per 100 cc. of blood in 6 weeks, while with an equivalent amount of iron as beef muscle or kidney there was little response. Our experience with beef muscle would indicate that copper was the limiting factor in the muscle as compared with the liver. The fact that we have obtained better results with 3 gm. of whole wheat furnishing only 0.1 mg. of iron than these investigators obtained with 0.15 mg. of iron which was largely in the form of hemoglobin; supports the view that the form of iron in liver and muscle is not as favorable for immediate use as the form in the cereal grains or in egg. This is further substantiated by the work of Sheets and Frazier (10) who found that daily portions of calf liver furnishing 0.25 mg. of iron, although rich in copper, gave an increase in hemoglobin of only 6 gm. in 6 weeks. There is ample evidence from the work of Mitchell and Miller (11, 12) and of Levine and his associates (13, 14) that 0.26 mg. of iron is sufficient for regeneration of 10 to 12 gm. of hemoglobin in 6 weeks when accompanied by sufficient copper; and we have found 0.19 mg. adequate for regeneration of this amount of hemoglobin in 6 weeks when administered in the form of whole wheat or bran.

With the accumulating evidence that copper influences the amount of hemoglobin formed from a given amount of iron, provided the iron is in a form readily available, the total amounts of

iron and copper ingested have taken on a new importance. The milk which we have used has by repeated analyses been found to have an iron content of about 0.8 mg. per liter and a copper content of about 0.27 mg. per liter. Using these figures to calculate the iron and copper intake from the milk, for which accurate consumption records have been kept, and adding the amounts ingested in the respective supplements, we have made estimates of total intake of iron and copper as indicated in the legends of Figs. 1 to 6. These data make it evident that hemoglobin regeneration can take place at the rate of about 10 gm. in 6 weeks when the young anemic rat is furnished a total of about 0.25 mg. of iron and 0.05 mg. of copper in milk plus a supplement whose iron and copper are readily available. But there are apparently certain factors in foods which prevent full hemoglobin regeneration. For example, white flour with added iron and copper to equal 6 gm. of whole wheat and also the hydrochloric acid solution of the ash of whole wheat gave an average increase of only 7.4 to 7.8 gm. respectively in 6 weeks as against 10 to 11 gm. with whole wheat or bran. These factors are now being made the subject of further study.

Milk in many instances furnished over 50 per cent of the total copper, although the copper content of our milk was fairly low. Eveleth, Bing, and Myers (15) report 0.30 to 0.38 mg. per liter in the milk used in their recent studies. It can be readily appreciated that better results may be obtained with foods poor in copper if the milk furnishes a still larger share of the intake. Thus Mitchell and Miller (11) found that rats receiving a water extract of spinach furnishing 0.25 mg. of iron and 0.007 mg. of copper regenerated hemoglobin to 14 gm. in 3 weeks, a gain of 8 gm.; but, as our rats on 0.20 mg. of iron and 0.007 mg. of copper needed a total of at least 0.03 mg. of copper to gain that much, milk seems to us the main source of their copper. Also the variability in the copper content of foods is large, as shown by the fact that while Mitchell and Miller report 0.007 mg. of copper in a portion of spinach to yield 0.25 mg. of iron, Levine, Culp, and Anderson report only 0.003 mg. of copper in a portion yielding 0.2 mg. of iron. In the latter case hemoglobin regeneration of 9.2 gm. per 100 cc. of blood was obtained in 6 weeks, which seems to us good evidence that their milk furnished considerably more copper than

## 228 Food Factors in Hb Regeneration. III

ours. It would seem important in further studies of foods to make a careful measurement of the total iron and total copper intakes, and not to note only the iron and copper in the supplement.

### SUMMARY

Rats reduced by exclusive milk feeding to a hemoglobin level of 4 to 6 gm. per 100 cc. of blood have been fed dried whole egg, dried egg yolk or its ash alone and supplemented by ferric chloride or copper sulfate or both, dried liver, fresh lean beef, prepared bran and its ash, and combinations of egg and whole wheat, egg and bran, and liver and bran, in a search for the factors in these foods influencing hemoglobin regeneration. The most important factor seems to be the total amounts of iron and copper, regeneration of 10 to 11 gm. in 6 weeks occurring only when the total iron is not less than 0.25 mg. per day and the total copper not less than 0.05 to 0.06 mg. But this amount of regeneration has been achieved on these levels of iron and copper only in the case of whole wheat, oatmeal, and prepared bran (56 animals). With the hydrochloric acid solution of the ash of each, or with egg yolk or its ash supplemented with copper (the first limiting factor in the egg), the hemoglobin regeneration has ranged between 8 and 9 gm. per 100 cc. of blood, and as the averages of the individual cases in the different series show a high probability of accuracy by "Student's" test (16), we believe that some additional factor is in operation to keep these returns on adequate iron and copper below the optimum. In liver, and to a lesser degree in egg yolk, the form in which the iron is held in the food is a limiting factor. It takes twice as much iron in the form of liver as of whole wheat, copper being adequate, to get equivalent regeneration. In the case of egg yolk, adding copper was much more beneficial than adding iron, but the results averaged 25 per cent higher with added iron than without it, and the difference between a given amount of egg yolk and the corresponding amount of iron and copper in the form of pure salts was in the same direction and somewhat greater. Hence the first limiting factor in egg yolk is copper, and the second appears to be the form in which the iron occurs. What the third may be is still under investigation.

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## EXCRETION OF AMMONIA AND NEUTRALITY REGULATION

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In a recent study of the acidosis of nephritis (1) attention was called to certain circumstances which were thought to throw some doubt on the prevailing belief that the excretion of ammonia into the urine serves as an important mechanism for conservation of fixed base and neutrality regulation in the tissues. It was also pointed out at the same time that all circumstances were consistent with the view that the ammonia excreted in the urine serves essentially to prevent excessive acidity in the lower urinary passages. That is, ammonia formation is stimulated by, and serves to neutralize the acid residue left in the tubules after resorption from the glomerular fluid of sodium chloride and sodium bicarbonate.

Some of the facts which seem most impressive are: Very little ammonia is excreted by a patient with advanced nephritis, yet there is usually no significant depletion of fixed base unless the patient is vomiting. Administration of a large dose of strong acid to a nephritic patient leads to no more waste of fixed base in the urine than from a normal individual, unless the patient should happen to have an abnormal store of extracellular fixed base in the form of edema. Diuresis in a normal man or animal prevents normal resorption of sodium bicarbonate. However, moderate diuresis produced in man by drinking distilled water fails to stimulate ammonia formation (2); and excessive diuresis with excessive waste of fixed base produced in animals by intravenous injection of neutral substances, such as urea or sodium sulfate, causes a marked decrease in the excretion of ammonia (3).

The evidence considered last above indicates that waste of fixed base alone is not an adequate stimulus for the formation of ammo-



		Man									
10	KCl, 5 gm.	66	10.86	0.89	3.42	15.17	+2.59	13.95	+4.65	2.31	2.45
11	" 5 "	89	12.65	2.01	3.10	17.76		18.60		3.86	1.81
12	" 5 "	90	23.40	1.82	4.15	29.37	+41.83	29.0	+53.50	2.04	2.37
		255	61.50	4.57	5.13	71.20		82.5		11.20	1.33
		460	24.60	2.12	3.90	30.62	+22.42	30.20	+28.50	2.38	4.98
13	Control	256	46.20	3.58	3.26	53.04		58.70		5.61	2.37
		102	21.20	1.93	3.67	26.80	-7.83	27.10	-8.22	1.63	3.57
14	" NaCl 5 gm.	82	12.65	3.20	3.12	18.97		18.88		1.60	3.54
		104	18.50	1.16	2.63	22.29	-2.69	20.90	-3.20	1.07	3.84
		74	16.20	1.07	2.33	19.60		17.70		0.79	3.45
15	Na <sub>2</sub> SO <sub>4</sub> , 3 gm.	68	13.60	1.54	3.60	18.74	+4.40	16.80	+3.10	1.26	3.22
		66	10.60	3.28	9.26	23.14		19.90		1.22	3.95
16	" 3 "	64	14.55	1.97	2.92	19.44	+2.56	17.45	+1.32	0.79	4.13
		61	9.46	5.36	7.18	22.00		18.77		0.77	4.52
17	" 3 "	100	15.85	2.21	2.52	20.58	+2.20	19.90	+1.70	2.16	3.33
		94	10.68	5.07	7.03	22.78		21.60		1.65	3.78



nia by the kidney. In the present contribution evidence will be presented which indicates that increased tissue acidity is likewise not an adequate stimulus.

#### EXPERIMENTAL

Studies were made concerning the influence of potassium chloride and of sodium sulfate on the excretion into the urine of the various acids and bases, when injected subcutaneously into dogs and when taken orally by man (Table I).

The work on dogs was done with males under prolonged urethane anesthesia, except in Experiment 7 in which nembutal was used. A flexible wax catheter was introduced and catheter drippings discarded for a half hour. A control urine was then collected for a period of 2 to 3 hours. At the end of this period the salt to be studied was injected in 5 per cent solution and another urine specimen collected over a period of the same duration as the control period. Collections were made more complete by pressure on the bladder at the end of each period. A few crystals of thymol were used as preservative in the collecting flasks. Some attention was directed to the rate and character of the respiration. No appreciable difference was observed between the two periods in any of the experiments.

The studies on man were started at about 11.00 a.m., at which time the bladder was emptied and a light lunch consisting of 20 gm. of strained honey, 20 gm. of plain cake, 20 gm. of cheese, and 200 cc. of tap water was consumed. The control urine was collected after a period of about 3 hours. At this time another lunch with the experimental salt incorporated in the honey was consumed and the second urine collected after an interval equal to that of the first period.

The chemical methods used for urine analysis were: for ammonia, the aeration technique of Van Slyke and Cullen; for chloride, a modified Volhard-Harvey titration. Phosphate was determined colorimetrically, hydroquinone being used as the reducing agent. The monovalent value was calculated since dibasic phosphate was part of the titratable alkali. Sulfate was determined by the method of Fiske; total fixed based, by the technique of Stadie and Ross (phosphate removed with iron). For the determination of titratable alkali an aliquot of urine was titrated to pH

4.8 with 0.1 N HCl from a micropipette, methyl red being used as indicator. A standard for comparison containing the same quantity of urine and indicator was adjusted with the aid of 0.5 M citrate buffer of pH 4.8. This titration was intended to show variations in excretion of bicarbonate and dibasic phosphate.

#### DISCUSSION

*Experiments with Potassium Chloride*—The results are shown in Experiments 1 to 4 and 10 to 12 inclusive. There is observed consistently an excretion of more extra fixed base than of extra mineral acid during the experimental period after injection of potassium chloride to dogs or ingestion of potassium chloride by man. The extra fixed base excretion is not simply a diuresis effect, for the subcutaneous injection of 30 cc. of 5 per cent potassium chloride in dogs under urethane anesthesia failed to produce a diuresis. Similar results are observed in one of the experiments on man in which an extra quantity of distilled water was ingested with the first lunch so as to make the volume of the control urine greater than that of the test period. At any rate the conditions of the experiments were such that a residue of acid was left in the tissues and, associated with this, there is invariably a decrease in the excretion of ammonia.

No significant change in excretion of ammonia had been observed in 24 hour metabolism experiments on white rats. Presumably, excretion of potassium and depression of ammonia formation took place in the early hours, followed by excretion of the extra chloride and increased formation of ammonia. Similar results from 24 hour metabolism experiments on man have recently been reported by Wiley, Wiley, and Waller (4). Retention of chloride, however, has been observed after 24 hours by Loeb, Atchley, and collaborators(5) in a patient with nephrosis; with retention of chloride the excretion of ammonia was decreased.

The increased excretion of ammonia following administration of acids or acid residue salts, such as  $\text{CaCl}_2$  or  $\text{NH}_4\text{Cl}$ , has ordinarily been looked on as a defensive mechanism for conservation of fixed base, stimulated in some unknown way by the acid residue in the tissues. Considerable doubt is thrown on such a view with the failure of ammonia stimulation by the acid residue left in the tissues after administration of potassium chloride.

*Experiments with Sodium Sulfate*—It has been clearly shown by Hendrix and Sanders (6) that the titratable acidity and ammonia content of the urine are increased after subcutaneous injections of dibasic sodium phosphate or sodium hippurate; and they point out that this is probably because sodium, a threshold substance, is more actively resorbed by the tubules. Since sulfate seems to be a most characteristic non-threshold substance, it should be possible to produce similar results with sodium sulfate, providing the accompanying diuresis is not too great.

The results of Experiments 6 to 9 and 15 to 17 inclusive show, with dogs subcutaneously injected, excretion of more extra mineral acid than fixed base, a slight decrease in titratable alkali, and a slight increase in excretion of ammonia. After oral ingestion by man the results are similar, although even less pronounced, possibly because sulfate is not so actively absorbed from the intestinal tract as sodium, or possibly because the quantity taken was restricted to a small dose in order to prevent laxation or diuresis.

It seems most improbable that increased ammonia excretion in these experiments with sodium sulfate, as well as those of Hendrix with dibasic sodium phosphate and sodium hippurate, could result from the change in the tissues. The obvious explanation is that the residue left in the tubules after resorption of sodium is more acid; ammonia formation results from this increased local acidity and serves to protect the lower urinary passages.

*Control Experiments*—It was not the object of this study to show that administration of potassium chloride would make the urine more alkaline or that sodium sulfate would make the urine more acid, but to see whether or not excretion of more extra mineral acid than fixed base would invariably stimulate ammonia excretion and *vice versa*. For this reason control experiments were not particularly valuable. However, one control experiment on a dog in which sodium chloride, the salt of a threshold acid and a threshold base, was injected shows little change in ammonia excretion and the extra excretion of fixed base is approximately equal to that of extra mineral acid. The two control experiments on man show similar results.

#### *Alkaline Reserve and Excretion of Ammonia*

In their study of diuresis, Hendrix and Calvin observed a marked drop in the  $\text{CO}_2$ -combining capacity of the blood associated with

waste of fixed base and decreased excretion of ammonia. In the study of Hendrix and Sanders, in which increased excretion of ammonia followed injection of dibasic sodium phosphate, there was undoubtedly an increase of  $\text{CO}_2$ -combining capacity. Excretion of ammonia may, therefore, vary in the same direction as the alkaline reserve, as well as the opposite direction, which is the usual case.

In the present study changes of the acid-base balance as indicated by urine analysis were usually not great; striking changes of the alkaline reserve could, therefore, not be expected. Two observations may be reported on plasma from venous blood saturated with atmospheric oxygen before separation. The plasma was saturated with alveolar air from a supply collected over dilute sulfuric acid. In Experiment 9 there was an increase from 63.3 to 65.3, and in Experiment 4 there was a drop from 60.7 to 59.8. These results are in the direction indicated by the changes in the acid-base balance of the urine; the associated changes of ammonia excretion in these experiments, like those of Hendrix and collaborators, cannot be explained by changes in the tissues.

#### *Rôle of Kidney in Neutrality Regulation of Tissues*

From the foregoing considerations it would seem that ammonia excretion bears no constant relation to acid-base changes in the tissues and that there is invariably a direct relation to the acid-base balance of the urine. Even so, it may be argued that fixed base would be excreted with extra mineral acid if ammonia were not substituted, so that after all excretion of ammonia operates as a base-conserving mechanism. The view taken by the present writer is that without ammonia excretion the urine would simply be more acid. It is interesting to observe that with impaired ammonia formation in nephritis the urine is more acid, and similarly in rabbits administration of strong acid causes the formation of a relatively acid urine with relatively little ammonia (7). The secretion into the urine of Prussian blue after injection of ferrocyanide and ferric ammonium citrate (8) indicates the formation somewhere in the kidney of a secretion more acid than is ever observed in the bladder, and suggests secretion of ammonia after secretion of acid.

This view is not in conflict with prevailing teachings of physi-

ology. The essential feature of neutrality regulation on an acid ash diet is resorption from the glomerular fluid of bicarbonate, along with water, glucose, and chloride. In states of alkalosis the tubules apparently reject bicarbonate.

#### SUMMARY

Following the administration of potassium chloride subcutaneously to dogs, or by mouth to man, the urine has been observed to become more alkaline because of excretion of more extra fixed base than mineral acid. Associated with the residue of acid thus left in the tissues, there has constantly been suppression of excretion of ammonia in the urine. Following administration of sodium sulfate, excretion of more extra mineral acid than fixed base with increased excretion of ammonia has been observed.

The results, considered with other observations, have been taken to indicate that the mechanism of ammonia excretion is not controlled by acid-base changes in the tissues.

All observations are in harmony with the view that ammonia excretion is stimulated by and serves to neutralize the acid residue left in the tubules after resorption from the glomerular fluid of the alkaline threshold moiety.

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# THE COMBINATION OF CARBON MONOXIDE WITH HEMOCYANIN\*

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Craifaleanu (1919), on bubbling carbon monoxide gas through hemocyanin solutions, observed that they turned colorless. When exposed to oxygen, the solutions apparently failed to regain color as rapidly as when reduced with an inert gas. Consequently, he thought it likely that a carbon monoxide compound of hemocyanin was formed which is unstable in the presence of oxygen. Dhéré and Schneider (1922) also observed that hemocyanin became colorless in the presence of carbon monoxide, and would recolor when exposed to air. Although they could see no difference between the action of carbon monoxide and an inert gas, they did not go so far as to conclude that there was no combination. Instead they stated that, if combination took place, the compound must be less stable than oxyhemocyanin, and that there was a need of quantitative data on the subject.

The data to be presented here indicate that carbon monoxide does combine with hemocyanin. Furthermore, they show that the combination contrasts sharply with that which takes place between carbon monoxide and hemoglobin, in that the carbon monoxide has a weaker, rather than a stronger, affinity for hemocyanin than has oxygen.

## Methods

*Limulus* serum preserved with a small amount of toluene was employed as a source of hemocyanin throughout the series of experiments. The serum was equilibrated in the Barcroft type of tonometer at a temperature of 25°. The gas phase in the tonometer was analyzed by means of the Haldane apparatus, to

\* Contribution No. 38.

which was attached a combustion chamber for the determination of carbon monoxide. The gas tensions were calculated by means of the equation of Bock, Field, and Adair (1924). The gases in the serum were measured by means of the Van Slyke constant volume apparatus to which was attached the special extraction chamber described by Redfield, Coolidge, and Hurd (1926). The gases were liberated from the serum in a manner described by Redfield, Coolidge, and Montgomery (1928), 2 cc. of 0.5 N KCN being used for 5 cc. of serum. Carbon dioxide was absorbed with 1 N sodium hydroxide, oxygen with sodium hydrosulfite, and carbon monoxide with ammoniacal cuprous chloride solution. In certain cases, on a duplicate determination, the carbon monoxide was not absorbed but was measured with the residual nitrogen, and its volume calculated by deducting the dissolved nitrogen. The pH of the serum was measured by means of the glass electrode.

#### *Solubility Coefficients of Oxygen and Carbon Monoxide*

The oxygen solubility coefficient has been calculated from the data of Fox (1909) for sea water, and of Redfield, Coolidge, and Montgomery (1928) for *Limulus* serum. The latter authors report a figure for the serum that is about 8 per cent lower than that for sea water at the same temperature. On the basis of this information and Fox's data for sea water at 25° and 18 gm. of chlorine per liter, a solubility coefficient of 0.022 cc. was adopted for oxygen at 25°.

In arriving at a suitable figure for the solubility coefficient of carbon monoxide it was assumed that the same relations between carbon monoxide and oxygen solubility would hold in serum as hold in pure water. According to Winkler (1901) carbon monoxide at 25° is 75.3 per cent as soluble as oxygen in water. On this basis the solubility coefficient for carbon monoxide in *Limulus* serum would be approximately 0.0166 cc. at 25°. In order to ascertain whether this value approximates the amount of gas in solution that can be measured with the technique employed, and thus afford a control for the main experiments, measurements were made on the amount of carbon monoxide taken up by sea water equilibrated with approximately an atmosphere of the gas. As a further check, the observation of Kobert (1903) that KCN prevents hemocyanin from combining with oxygen was made use of.

It seemed not unlikely that KCN might also prevent hemocyanin from combining with carbon monoxide. Consequently 1.7 cc. of a 10 per cent KCN solution were added to 30 cc. of serum. Samples of the resulting solution were then equilibrated with carbon monoxide and the gas in the serum measured. Data for both sea water and KCN-serum are recorded in Table I. It can be seen from these data that the use of 0.0166 cc. as a solubility coefficient introduces no great error, since it approximates actual measurements. The value for the KCN-serum, though slightly higher than that for sea water, indicates that serum treated with KCN does not combine with carbon monoxide.

TABLE I  
*Carbon Monoxide Dissolved in Sea Water and KCN-Serum at 25°*

Sample	$P_{CO}$	CO content*		Solubility coefficient†
		(a)	(b)	
	<i>mm. Hg</i>	<i>vol. per cent</i>	<i>vol. per cent</i>	<i>cc. per cc.</i>
Sea water.....	750	1.64	1.77	0.0166
	759	1.68	1.76	0.0168
KCN-serum.....	710	1.60	1.78	0.0171
	712	1.61	1.80	0.0172

\* In column (a) the values were obtained by absorption with cuprous chloride. In column (b) they were obtained by measurement of residual gas and by deducting dissolved nitrogen. See text.

† The solubility coefficients are calculated on the basis of the figures in column (a) only.

#### *Carbon Monoxide-Combining Power of Hemocyanin*

Undialyzed serum was equilibrated with approximately an atmosphere of carbon monoxide, and with lower gas tensions. The gas estimated to be dissolved was then subtracted from the total gas found in the serum. Table II contains the results obtained. In column (a) the figures represent the carbon monoxide values obtained by absorption with cuprous chloride. There was some question as to whether all the gas was being absorbed on account of the fact that there seemed to be too much residual gas left. In order to obtain data by a means other than absorption, as a check on the method, in certain cases the volume of all the gas other than carbon dioxide and oxygen was measured and the carbon monoxide



## 242 Combination of CO with Hemocyanin

calculated by deducting dissolved nitrogen. The data obtained by this method are shown in columns (b) of Table II. This procedure was nearly always accompanied by a determination by absorption. There is a peculiar discrepancy between the figures obtained by the two methods that seems greater than could be accounted for on the basis of incomplete absorption. What is the cause of the discrepancy was not ascertained. However, it does not invalidate the conclusions one can draw from the experi-

TABLE II  
*Combination of CO with Hemocyanin at 25°*

The sample of *Limulus* serum used had an O<sub>2</sub> capacity of 1.17 volumes per cent.

P <sub>CO</sub>	P <sub>O<sub>2</sub></sub>	CO content*		CO combined*		O <sub>2</sub> content	O <sub>2</sub> combined
		(a)	(b)	(a)	(b)		
mm. Hg	mm. Hg	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent
727	16.0	2.39		0.80		0.42	0.37
743		2.30		0.68		0.30	
758	6.0	2.52		0.96		0.11	0.09
(700)†	1.8		2.75		1.22	0.11	0.11
(700)	7.3	2.55		1.02		0.12	0.10
(700)	2.4	2.59		1.06		0.12	0.12
696	8.0	2.35	2.70	0.83	1.18	0.08	0.06
724	11.6	2.43	2.67	0.85	1.09	0.15	0.12
178	5.0	0.85		0.46		0.26	0.25
164		0.75		0.39		0.10	

\* See asterisk foot-note to Table I for explanation of columns (a) and (b).

† Not obtained by analysis; tonometers filled with approximately this amount of gas.

ments. The data clearly indicate a significant amount of carbon monoxide in the serum above that dissolved. If we compare them with the figures obtained with sea water and KCN-serum, the difference is very evident.

As a further test some of the serum was concentrated by dialysis so that the oxygen capacity was increased to a value about 4 times greater than the original. A sample of this serum equilibrated with about 700 mm. of carbon monoxide and a small amount of

oxygen gave a value for combined carbon monoxide of 3.3 volumes per cent and for oxygen of 0.94 volume per cent.

It will be noted in Table II that a small amount of oxygen in the presence of a large amount of carbon monoxide is still quite effective in combining with hemocyanin. This observation made it seem worth while to compare an oxygen dissociation curve established in the presence of carbon monoxide with one obtained in the

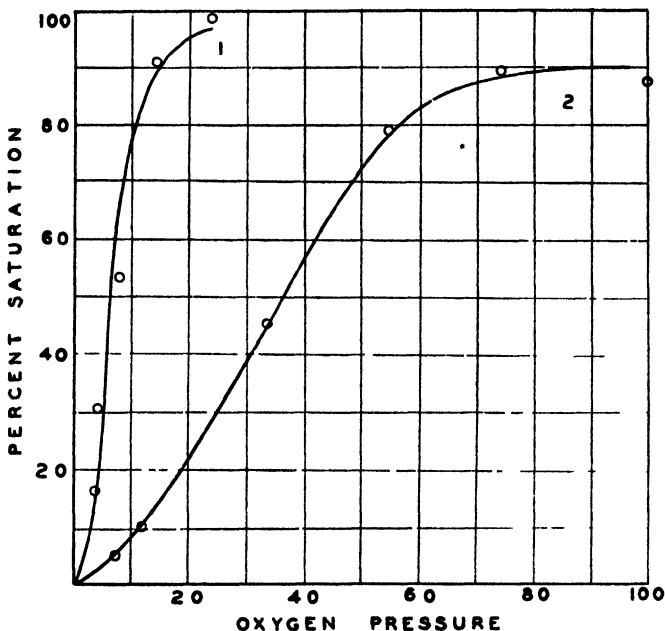


FIG. 1. Oxygen dissociation of *Limulus* serum. Curve 1 was established in the total absence of carbon monoxide. Curve 2 was established in the presence of about 700 mm. of carbon monoxide. The pH of the serum was 7.5 in both cases.

total absence of the gas. If the two curves obtained under such conditions are not superimposed, but the one in the presence of carbon monoxide is displaced to the right, it will not only be clear that carbon monoxide is replacing some of the oxygen in its combination with hemocyanin, but one will also be able to calculate the combining ratios of the two gases. The data for this study are summarized in Fig. 1. They clearly confirm the fact that carbon

monoxide combines with hemocyanin. Furthermore, the data for the dissociation curve in the presence of carbon monoxide indicate that there is sufficient carbon monoxide present to saturate the hemocyanin when there is little oxygen present. Therefore, the combining ratios of the two gases can be calculated by reading from the curve the respective pressures of the two gases at a point where each is combined with just half of the hemocyanin. It will be noted that this condition obtains at 35 mm. pressure for oxygen and about 700 mm. pressure for carbon monoxide. The combining ratios are, therefore, as 1:20; *i.e.*, oxygen has an affinity for hemocyanin that is about 20 times greater than that of carbon monoxide. This is surprising when one considers the relative affinities of these two gases for mammalian hemoglobin.

It would appear from the data presented that in carboxyhemocyanin the same stoichiometrical relation exists as does in the case of oxyhemocyanin; namely, 1 molecule of the gas is bound for each 2 atoms of copper present in the hemocyanin.

#### SUMMARY

Carbon monoxide combines with hemocyanin. The compound formed is less stable than oxyhemocyanin, the affinity of the gas for *Limulus* hemocyanin being only about one-twentieth the oxygen affinity. The same stoichiometrical relation exists in the compound as does in the case of oxyhemocyanin; namely, 1 molecule of the gas for each 2 atoms of copper.

I wish to thank Professor A. C. Redfield at whose suggestion this investigation was undertaken. I wish, also, to thank Mr. Walter Buck for technical assistance during the early part of the work.

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# THE SOLUBILITY AND PREPARATION OF PHOSPHORUS- AND NITROGEN-FREE GLYCOGEN

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Available data on the solubility of glycogen in alcohol-water mixtures are chiefly concerned with conditions which insure the complete precipitation of the substance for the purpose of quantitative determination. The comprehensive work of Kerly<sup>1</sup> in this field deals extensively with the solubility of glycogen in mixtures containing 55 to 90 per cent alcohol, but contains no information in regard to lower alcohol concentrations. Our interest lay in the latter, mainly from the point of view of the preparation and isolation of glycogen in pure form.

It is known that highly refined glycogen preparations, even after repeated electrodialysis, tenaciously retain variable amounts of phosphorus and nitrogen so that some investigators believe these elements to be integral constituents of the glycogen molecule. We took as a working hypothesis the opposite view; namely, that phosphorus and nitrogen, at least in part, are constituents of admixed organic colloids which in their response to dialysis and electrophoresis resemble glycogen. Likewise, it was assumed that these admixtures are precipitated from aqueous alcohol together with glycogen, regardless of the number of reprecipitations, so long as the conventional concentration of alcohol, *i.e.* 2 volumes of 95 per cent alcohol to 1 volume of aqueous glycogen solution, is employed. We were looking, therefore, for the minimum alcohol concentrations which would suffice to precipitate glycogen with little or no loss, assuming the possibility that at these concentrations contaminating admixtures might remain in solution, wholly or in part. It may be stated that our experiments have borne out this assumption.

<sup>1</sup> Kerly, M., *Biochem. J.*, **25**, 671 (1931).

*Solubility*

The glycogen used at the outset of this work was a preparation obtained from dog liver by Pfüger's method. Empirically refined by fractional precipitations at alcohol concentrations below 50 per cent, the final product contained 0.03 per cent nitrogen and 0.002 per cent phosphorus and was entirely free of electrolytes. Later we employed material, prepared by the method to be pre-

TABLE I

*Showing Solubility of Glycogen at Various Alcohol and NaCl Concentrations at 22° and 0°*

NaCl	Alcohol, volumes per cent							
	26	30	34	38	39	43	47	50
	Glycogen in solution per 100 cc mother liquor							
At 22°								
M	mg	mg	mg	mg	mg	mg	mg	mg
0 005					4	1	0	0
0 010	>2500	>2500	±1000		0 5	0 5	0	0
0 050					0 6		0	0
0 100						1	0	0
0 250					3	1	0	0
0 500					15	2	0	0
0 750					26	1	0	0
1 000	>2500	>2500	>2500	>1000	32	2	0	0
At 0°								
0 005					0 4	0	0	0
0 010					0 4	0	0	0
0 020	38	14			0 4	0 5	0	0
0 100					1	0 2	0 5	0 3
1 000	680	310	200	12	5	1	0 5	0 3

sented in this paper, which contained no measurable amounts of either phosphorus or nitrogen.

The effect of alcohol concentration, of electrolytes, and of temperature upon the solubility of glycogen was examined by the following technique: To 5 cc. portions of a 10 per cent aqueous glycogen solution in a series of 25 × 200 mm. Pyrex test-tubes electrolyte solutions and diluted alcohol were added in calculated

amounts so as to yield the desired concentrations of electrolyte and of alcohol in an ultimate volume of 20 cc. (The mixture contained 2.5 gm. of glycogen per 100 cc. where no precipitation has occurred.) The tubes were stoppered, immersed in a water bath of 4 gallons capacity at a temperature of 35°, then the temperature was gradually lowered by blowing a continuous current of air over the surface of the water until a stable temperature was reached and could be sustained over a period of 12 to 14 hours. At that time the tubes were centrifuged, the supernatant fluid was decanted, and the glycogen determined as glucose in the well drained precipitate and in certain instances also in the mother liquor.

An identical set of tubes was immersed in ice water, placed in the refrigerator, and kept at 0° for 12 to 14 hours, after which period glycogen determinations were carried out as in the preceding group.

*Effect of Alcohol Concentration*—The results of such a series of experiments are presented in Table I. Considering alcohol concentration as the first among the three factors which affect the solubility of glycogen, it may be seen that at 22° about 1 gm. per 100 cc. of liquid is soluble when the alcohol concentration is as low as 34 per cent; from this point upward the solubility declines rapidly until somewhere between 43 and 47 per cent alcohol concentration 100 per cent of the glycogen is precipitated. Proof of complete precipitation is the fact that the mother liquor, after evaporation of its alcohol content and subsequent hydrolysis with 0.6 N HCl, yields no reducing matter whatsoever. (The findings of Kerly that slight amounts of glycogen are kept in solution even at 60 and 80 per cent alcohol concentration, are in apparent contradiction to our results. But it must be noted that Kerly has not attempted to discriminate between glucose and reducing substances other than sugar, and, furthermore, that she employed a ferricyanide reagent which is very sensitive to non-sugar reducing substances.)

*Effect of Sodium Chloride*—Unexpected was the influence of the electrolyte (in this experiment sodium chloride), inasmuch as increasing concentrations of it increased the solubility of glycogen. Thus, in 100 cc. of 34 per cent alcohol only about 1 gm. of glycogen was soluble when the NaCl was in 0.01 M concentration; whereas, all of the glycogen present, 2.5 gm. per 100 cc., was kept in solution

when the concentration of the salt was raised to molarity. As the alcohol concentration reached 43 per cent, however, the effect of variations in the salt concentration was scarcely perceptible and was entirely ruled out at alcohol concentrations exceeding 43 per cent.

*Effect of Temperature*—This may be simply characterized by stating that up to the critical level of alcohol concentration,

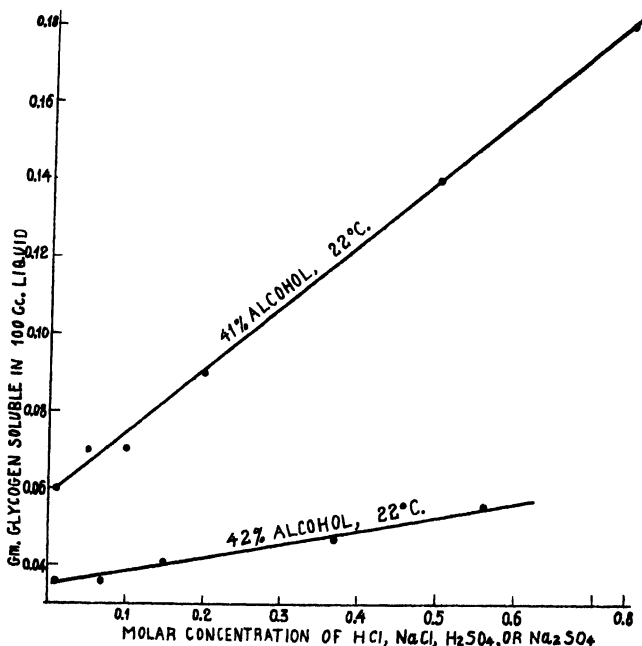


FIG. 1. Effect of varying concentrations of chloride and sulfate upon the solubility of glycogen in alcohol-water mixtures.

around 45 per cent, the lowering of temperature is analogous in its effect to increasing the alcohol concentration. Thus at 0°, the precipitation of glycogen is nearly complete when the alcohol content is 39 per cent, and complete with 43 per cent alcohol, at least with favorable, low electrolyte concentrations. With 30 per cent alcohol, at 0°, the solubility of glycogen is only 14 mg. per 100 cc.; whereas, at 22°, the entire amount present, 2.5 gm. per 100 cc., is held in solution. Decrease in the temperature also dimin-

ishes the salt effect in the same manner as does a rise in the alcohol concentration.

*Comparison of Effect of Chloride and Sulfate Ions*—Another group of experiments served to gage accurately the salt effect at alcohol concentrations of 40 and 42 per cent, and at the same time to compare the effects of  $\text{NaCl}$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{HCl}$ , and  $\text{H}_2\text{SO}_4$ . It was found that the actions of these four compounds in equimolecular concentrations are identical. These results are presented in Fig. 1. The two curves show that the salt effect diminishes quite sharply with but 1 per cent rise (from 42 to 43) in the alcohol content, as the point of critical alcohol concentration is approached; beyond

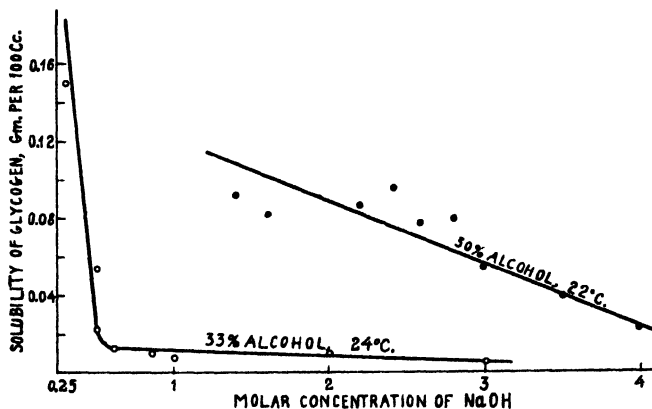


FIG. 2. Effect of varying concentrations of  $\text{NaOH}$  upon the solubility of glycogen in alcohol-water mixtures.

this, variations in the salt concentration no longer affect the solubility of glycogen.

*NaOH As Electrolyte*—Particular interest must attach to the influence as an electrolyte of caustic alkali because of its use in the preparation of glycogen. The results of extensive experiments dealing with the effect of varying concentrations of  $\text{NaOH}$  and alcohol upon the solubility of glycogen are condensed in Fig. 2. It may be seen that, contrary to the behavior of chlorides and sulfates in regard to their influence upon the precipitation of glycogen, the efficacy of  $\text{NaOH}$  gradually increases as its concentration rises. As a consequence, the precipitation of glycogen is as efficacious with lower concentrations of alcohol in the presence



of much NaOH, as with higher alcohol concentrations and less NaOH. Thus, when the alcohol content of the solvent is as low as 30 per cent, the concentration of NaOH must be 4 N in order to effect a nearly complete precipitation (about 20 mg. of glycogen per 100 cc. of fluid is held in solution); whereas, about 0.5 to 1.0 N NaOH suffices when the alcohol concentration is raised to 33 per cent (9 mg. of glycogen per 100 cc. of solvent remain in solution). It is noteworthy that the solubility of glycogen in 33 per cent

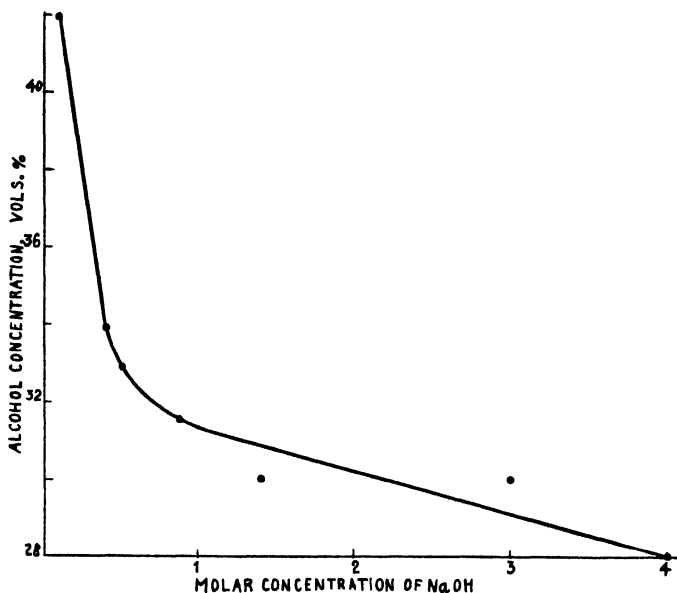


FIG. 3. Coordinated concentrations of alcohol and NaOH at which the solubility of glycogen is less than 0.1 gm. per 100 cc. of solvent.

alcohol increases sharply when the concentration of NaOH falls below 0.5 N.

From the point of view of glycogen preparation we have determined for a series of NaOH concentrations the corresponding minimum alcohol concentrations which suffice to effect precipitation without appreciable loss.

The data are given in Fig. 3; the curve represents coordinated alcohol and alkali concentrations at which the solubility of glycogen is considerably below 0.1 gm. per 100 cc. of the solvent. It may

be seen that this end is attained with 42 per cent alcohol in the presence of about 0.1 N NaOH, and also *with only 28 per cent alcohol* when the concentration of the alkali is 4 N. Again, an abrupt break appears between the alcohol concentrations of 31 and 34 per cent, indicating that in order to accomplish the complete or nearly complete precipitation of glycogen, the concentration of the alkali must be increased to normality and above whenever the mother liquor contains less than 34 per cent alcohol.

### *Preparation*

Modifications, based upon the foregoing findings, may be incorporated with advantage in any of the numerous procedures for the preparation of glycogen. We have employed low alcohol concentrations in several variations of Pflüger's method, with the uniform result that the products were nearly or entirely free of phosphorus and nitrogen. In the following we describe the preparation of glycogen from liver by a procedure which for the time being is the most satisfactory in our hands.

Introduce the ground liver into a flask, add 2 cc. of concentrated (50 to 60 per cent) NaOH for each gm. of tissue, immerse in boiling water, and heat for 3 hours with occasional agitation during the 1st hour. Upon cooling without disturbance, a cake of soap congeals on the surface, occluding all solid particles, and the perfectly clear, gelatinous fluid can be poured off. (Hydrolysis with 60 per cent KOH, followed by salting out with NaCl, leads to the same result.) To the soap in the flask add water about equal in volume to the decanted fluid, heat until the soap is evenly distributed, then add NaCl until it again separates out, cool, and filter. Unite the two fluids, measure the volume, and introduce with continuous agitation *50 cc. of 95 per cent alcohol for each 100 cc. of alkaline fluid.* This results in an alcohol concentration of *33 per cent.* Allow the precipitate to settle overnight at room temperature, syphon off the clear layer of the supernatant fluid, and from the rest separate the glycogen by centrifugation or filtration. Prepare a washing solution by adding 1 volume of alcohol to 2 volumes of a 20 per cent NaOH solution; wash on the filter or in the centrifuge tube until the washing fluid is colorless or nearly so, and perform a last washing with 95 per cent alcohol. (The use of the centrifuge in this operation insures speed and

efficacy, since the precipitate can be thoroughly stirred and mixed with the washing fluid.)

Dissolve the impure glycogen in water while still moist with alcohol and remove the insoluble particles by filtration. To the filtrate add 2 N HCl until it gives distinct but not maximum acid reaction (brownish blue color) with Congo paper. Some precipitate separates out. Measure the volume of the solution and add 50 cc. of 95 per cent alcohol for each 100 cc. in order to bring about a better flocculation of the precipitate. Filter, returning the first fractions of the filtrate on the filter paper. Add to the filtrate more 95 per cent alcohol, so as to increase its total amount to 80 cc. for each 100 cc. of the original acid aqueous solution. This results in *an alcohol concentration of approximately 45 per cent, sufficient to effect the complete precipitation of glycogen in the acid medium.* Allow the precipitate to settle for a few hours, or overnight if convenient, separate the precipitate by centrifugation or filtration, and wash consecutively twice with 45, twice with 95 per cent alcohol, and finally with ether. The strong alcohol and the ether are designed mainly for dehydration; and to further this effect, stir and finely distribute the glycogen in these liquids by means of a glass rod. After the last washing, place the centrifuge tube at a slant, mouth down, to enhance the evaporation of ether. The glycogen soon can be poured from the tube in the form of a fine white powder and should be left exposed to air to allow the complete evaporation of alcohol and ether.

The product thus obtained still contains an appreciable amount of NaCl, and as a rule two reprecipitations with 45 per cent alcohol are required for its removal. If in the final precipitation the alcohol causes only milky opacity but no flocculation, owing to the absence of electrolytes, addition of 0.3 cc. of 0.1 N HCl per 100 cc. of fluid suffices to effect immediate precipitation, and the glycogen may be separated and dried as described.

It is advisable to redistil the commercial 95 per cent alcohol and also the ether to be used in the procedure in order to eliminate impurities which might be adsorbed by glycogen. The danger of it is illustrated in Kerly's work, in which the phosphorus content of a preparation was increased by repeated precipitations with alcohol.

Analysis shows our preparations to be free of phosphorus and

nitrogen, and to contain no measurable amount of inorganic matter. The aqueous solutions are perfectly clear and transparent in transmitted light, and opalescent, but never milky opaque, in reflected light even in concentrations of 5 to 10 per cent. Upon hydrolysis with acid no trace of yellow color and none of the familiar flimsy precipitate occur either in the acid state or after neutralization.

As may be seen, we attribute our success in isolating this pure product primarily to the avoidance of the conventional high alcohol concentrations. When the alcohol concentration (33 per cent) in our first alkaline mother liquor is increased to 60 to 70 per cent after the glycogen had been removed, additional precipitate is formed. Analysis of this shows that it consists mainly of inert matter with a relatively high phosphorus content, while the glycogen in it is but 5 to 10 mg. per 100 cc. of the original mother liquor.

Similar conditions prevail at precipitation from acid solution: At the low alcohol concentration employed (45 per cent), and at definitely acid reaction, some material other than glycogen is held in solution; this is precipitated from a neutral solution and is partially precipitated even from an acid medium when the alcohol concentration is increased.

#### SUMMARY

The solubility of glycogen in water-alcohol mixtures was studied with a view to finding the lowest alcohol concentrations applicable in the preparation and purification of glycogen. These experiments furnished the foundation for a procedure by which nitrogen- and phosphorus-free glycogen is obtained.



# THE ISOLATION AND DETECTION OF BILIRUBIN

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(Received for publication, November 15, 1933)

Since the authors have been working over a prolonged period on the isolation and detection of small amounts of bilirubin, and the work of Daddi (1) and Laemmer and Beck (2) along similar lines was reported recently in the literature, it seemed expedient that we report the results we had obtained.

Besides using  $\text{BaCl}_2$  and  $\text{Na}_2\text{CO}_3$  as bilirubin precipitants, we have tried  $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ ,  $\text{MgCl}_2$ ,  $\text{ZnSO}_4$ ,  $\text{Al}_2(\text{SO}_4)_3$ ,  $\text{CaCl}_2$ ,  $\text{FeSO}_4$ ,  $\text{FeCl}_3$ ,  $\text{CuSO}_4$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{Na}_2\text{HPO}_4$ , and  $\text{Na}_3\text{PO}_4$  respectively with varying amounts of  $\text{Na}_2\text{CO}_3$ . Of all the precipitants tried, best results were obtained by using a mixture of  $\text{BaCl}_2$  and  $\text{Na}_2\text{HPO}_4$  or  $\text{BaCl}_2$  and  $\text{Na}_3\text{PO}_4$ . (Mixtures of  $\text{BaCl}_2$  and  $\text{NaH}_2\text{PO}_4$  were found to be of no value.) For example, 6.0 cc. of  $\text{M}$   $\text{BaCl}_2$  solution and 6.0 cc. of  $\text{M}/3$   $\text{Na}_2\text{HPO}_4$  solution (5 per cent) united with 1.0 cc. of bile diluted 1 : 10 showed as much recovered bilirubin as a solution of 6.0 cc. of  $\text{M}$   $\text{BaCl}_2$  and 6.0 cc. of  $\text{M}$   $\text{Na}_2\text{CO}_3$  added to 1.0 cc. of bile previously diluted 1 : 1 with water. We usually filtered the solutions, extracted the residues with dilute  $\text{HCl}$ , and left the residue of bilirubin on the paper until dry. Extraction with hot alcohol yielded a filtrate containing bilirubin and biliverdin.

To detect the bilirubin we tried other diazo chlorides than *p*-sulfobenzene diazo chloride, first suggested by Ehrlich (3), later used by Pröscher (4), and applied by van den Bergh (5) for the detection of obstructive jaundice. We carefully diazotized the following amines: aniline, *o*- and *p*-toluidines, commercial xyldine, J acid, H acid, dianisidine, amino G acid, benzidine, *o*-, *m*-, *p*-anisidines, naphthionic acid, anthranilic acid, *p*-nitroaniline, 5-aminosalicylic acid,  $\alpha$ - and  $\beta$ -naphthylamines, and sulfanilic

acid. The diazo chlorides were prepared with KI-starch paper, any excess of nitrous acid being avoided.

On the addition of the respective diazo chloride solution to the neutral alcoholic solution of bilirubin, pronounced colors developed in only two cases. The diazo chloride from  $\alpha$ -naphthylamine gave a most intense violet color but it was found that the addition of the diazo chloride to ethanol alone gave the same result. We have not been able to explain this fact. The diazo chloride from sulfanilic acid gave the usual violet-red color associated with the van den Bergh test. It seemed unusual that of the many diazo chlorides tried, only one would be found to react with bilirubin to give a highly colored product.

While our work was in progress, Greco (6) published an important paper that had in it several new and valuable hints. We have used his method with very good results. We were unable to duplicate that part of his work where he matched the resulting colored solutions with solutions of mixed indicators. We found that the extraction of the barium phosphate-bilirubin precipitates with alcoholic NaOH shortened the method and thus prevented an appreciable oxidation of bilirubin to biliverdin. In place of 0.1 M alcoholic NaOH used by Greco, we used a solution of 0.5 gm. of sodium in 100 cc. of 95 per cent alcohol since it was less colored. We found that the alcoholic NaOH did not extract all the bilirubin from the precipitate and that it was better to dissolve the precipitate in glacial acetic acid which was a good solvent for both the bilirubin and the phosphate. We were agreeably surprised to find that on adding the *p*-nitrobenzene diazo chloride reagent to the strong acetic acid solution, an intense violet color developed. On the addition of the  $H_2SO_4$ , we centrifuged the mixture and made comparisons on the part poured off from the compact mass of  $BaSO_4$ .

We preferred the diazo chloride produced from *p*-nitroaniline as a bilirubin reagent on account of its stability. In this laboratory, Snow (7) and the senior author showed that the diazo chloride from *p*-nitroaniline was much more stable than the one from sulfanilic acid. When bilirubin in alcoholic NaOH solution was treated with *p*-nitrobenzene diazo chloride solution, a pronounced red-violet color developed and this became much more intense on the addition of the  $H_2SO_4$ . The color reached its maximum in about 20 minutes and persisted unchanged for several days.

## SUMMARY

1. Bilirubin is best precipitated from a dilute aqueous solution by means of  $\text{BaCl}_2$  and  $\text{Na}_2\text{HPO}_4$  or  $\text{BaCl}_2$  and  $\text{Na}_2\text{PO}_4$ .

2. In neutral alcoholic solution, bilirubin reacted only with *p*-sulfo-*benzene* diazo chloride to give a highly colored product.

3. In an alkaline alcoholic solution of bilirubin, both *p*-sulfo-*benzene* diazo chloride and *p*-nitrobenzene diazo chloride reacted.

4. In an acetic acid solution of bilirubin, *p*-nitrobenzene diazo chloride yielded a highly colored product.

5. The colors produced were pronounced and were stable for days.

6. Biliverdin showed no evidences of reacting with diazo chlorides.

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## THE CHEMICAL NATURE OF RENNIN

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(Received for publication, December 7, 1933)

One of the oldest controversies in physiological chemistry is the one concerning pepsin and rennin. The similarity in the mode of action of these enzymes seemed to make their separation and differentiation impossible; and the earlier workers used a variety of indirect methods to prove their contentions. Confusion was increased by incorrect statements, even up to quite recent times. For example, Edie (1) claimed to have obtained pepsin free of milk-coagulating power; whereas, it is quite evident that this cannot be the case since even highly purified pepsin has a rather high milk-clotting value (2, 3), although not as high as rennin. Differences in the inactivation temperatures and in the sensitivity to an alkaline reaction were also erroneously stated to be characteristic for these proteases (4).

Many efforts have been made to separate the two enzymes. As was to be expected, the adsorption method was tried but was unsuccessful (5). We have recently reported the isolation of a rennin preparation which was more active than any previously described and which was free of peptic activity. The method of preparing this pepsin-free rennin was dialysis and fractional isoelectric precipitation. The composition and chemical properties indicate that the enzyme is a thioprotease. Our findings have been verified by Holter (3) working in Sørensen's laboratory. We have also shown (6, 7) that the zymogens of rennin and pepsin differ in many respects and that there is no rennin in the adult mammalian stomach. In 1927 von Euler (8) remarked: "Der endgültige Beweis, dass das echte Lab und Pepsin ganz verschiedene Enzyme sind, wäre die präparative Trennung oder die Zerstörung des einen Enzymes unter Erhaltung der Wirkung

des anderen." With this in mind we have again taken up the study of rennin. We are reporting on experiments in which we have undertaken the digestion of rennin with pepsin, trypsin, and erepsin. These tests support our earlier work in which it was shown that rennin is a protein.

The second part of our paper deals with the absorption of rennin on crystalline edestin. In recent papers Dyckerhoff and Tewes (9) and Waldschmidt-Leitz and Kofranyi (10, 11) report experiments which indicate that crystalline enzyme preparations may be merely crystalline proteins which have adsorbed the enzyme. For example, they have compared the activity of crystalline pepsin, prepared according to Northrop (12), with edestin and other plant proteins which had been in suspension in pepsin solutions. The latter were found to be just as active as the former and, according to these workers, the active principle may exchange one protein carrier for another. Northrop (13) has repeated the work of Dyckerhoff and Tewes and that of Waldschmidt-Leitz and Kofranyi on the absorption of pepsin by crystalline proteins and found that, "The pepsin protein is taken up as such and the quantity of protein taken up by the foreign protein is just equivalent to the peptic activity found in the complex." The present work was completed before the publication of Northrop's paper.

Dyckerhoff and Tewes also state that rennin may be obtained similarly, that is, absorbed on crystalline edestin, and thus appear to be a highly active crystalline enzyme. However, they give no experimental data to substantiate this.

We were particularly interested in the rennin phase of their work for reasons stated above. Using methods similar to those Dyckerhoff and Tewes, and Waldschmidt-Leitz and Kofranyi employed with pepsin, we have attempted to obtain edestin-rennin crystals similar to those mentioned by them.

## EXPERIMENTAL

### *Digestion Experiments*

*Digestion of Rennin by Pepsin*—Highly active rennin was prepared according to our method (2) from the fourth stomach of the calf. The rennin had a milk-clotting power of 4,425,000 units per mg. A unit is the amount of milk in mg. clotted by 1 mg. of

rennin at 40° in 10 minutes. 10 cc. samples of CaCl<sub>2</sub>-milk (2) of pH 6.2 were used. For the digestion of rennin by pepsin a 0.2 per cent solution of rennin in 0.01 N HCl, with 8,850,000 units per cc., and a 0.5 per cent solution of pepsin (Parke, Davis and Company, 1:3000)<sup>1</sup> in 0.01 N HCl, with 1,700,000 units of rennet activity per cc., were employed. To 10 cc. of the rennin solution, 10 cc. of pepsin solution were added and incubated at 40° (Table I). As a control an equal volume of boiled pepsin solution was added to a sample of rennin; and samples of rennin and pepsin, respectively, were also kept at 40° for the same length of time. All samples were adjusted at the start of the experiments to pH 2.3 with HCl. Toluene was used as an antiseptic.

In Table I, Column 2 shows the total rennet activity of the pepsin-rennin mixture. If the milk clotting due simply to the

TABLE I  
*Showing Rapid Digestion of Rennin by Pepsin*

Hrs. at 40° (1)	Total rennet activity per cc. (2)	Rennet activity of pepsin per cc. solution (3)	Rennet activity of rennin per cc. solution (4)
	<i>units</i>	<i>units</i>	<i>units</i>
0	5,275,000	850,000	4,425,000
3	2,110,000	850,000	1,260,000
6	1,020,000	850,000	170,000
18	840,000	840,000	None
24	840,000	840,000	"

pepsin (Column 3) is subtracted, the figures of Column 4 are obtained. They indicate the number of units of rennet activity attributable to the rennin of the mixture and show that more than 90 per cent of the rennin has been digested in 6 hours and 100 per cent in 18 hours or less. Experiments with Northrop's crystalline pepsin yielded similar results.

*Digestion of Rennin by Trypsin*—A 0.3 per cent solution of trypsin (Fairchild Brothers and Foster) was made in 0.1 N acetate of pH 6.2 and a 0.2 per cent solution of rennin in 0.1 N acetate buffer of 6.2. For the digestion equal volumes were mixed to-

<sup>1</sup> An enzyme solution to be used for the digestion of another enzyme should not be too concentrated, since it may act as a protective colloid, preventing or retarding hydrolysis. We have first described this interesting phenomenon in 1931 (14).

gether. To one sample of rennin solution boiled trypsin solution was added, and samples of rennin and trypsin, respectively, in acetate buffer, were kept separately at 40° to serve as controls. All samples were adjusted to pH 6.2 with acetic acid at the start of the experiment. The rennin solution contained 8,850,000 units per cc. The trypsin solution was too dilute to clot milk or to influence rennet activity in any way. There was no appreciable change in the hydrogen ion concentration of the various samples at the end of the experiment. An acid pH was chosen because of the sensitivity of rennin to alkalinity or neutrality.

TABLE II  
*Digestion of Rennin by Trypsin*

Hrs at 40°	Units of rennin per cc.
0	4,425,000
3	330,000
6	1,000
10	None

Determination of the rennet activity after 3, 6, and 10 hours digestion by trypsin showed (Table II) that the digestion of rennin by trypsin is even more rapid than with pepsin. After 6 hours, less than 1 per cent of rennin is left. The digestion of rennin is complete in less than 10 hours, the mixture completely losing its power to clot milk.

*Digestion of Rennin by Erepsin*—A 0.3 per cent solution of erepsin (Digestive Ferments Company) was made in 0.1 N acetate buffer of pH 5.8 and also a 0.2 per cent solution of rennin in 0.1 N acetate buffer of pH 5.8. A boiled sample of erepsin served as a control. At the beginning of the experiment the samples were adjusted to pH 5.8 with acetic acid. The activity of the rennin solution was the same as that of those used in the other digestion experiments.

In contrast to the quick digestion of rennin by pepsin and trypsin, none of the rennin was digested by the peptidase erepsin within 24 hours.

#### *Absorption of Rennin by Edestin*

*Experiment 1*—This experiment was carried out according to the directions of Dyckerhoff and Tewes for pepsin. A 2 per cent

solution was prepared by dissolving the rennin (Parke, Davis and Company, 1:30,000, containing 22 per cent NaCl) in 0.2 per cent HCl and adjusting it to pH 7.0 with ammonia. 50 cc. of this rennin solution were cooled to 0° and 2.5 gm. of crystalline edestin, which had been suspended in 10 cc. of distilled water, cooled to 0°, were added to it. The mixture was kept at 0° for 15 minutes. The crystals of edestin showed slight changes under the microscope. After 15 minutes standing the edestin was centrifuged and washed twice with 10 to 20 cc. of water. The crystals were dissolved in 0.2 per cent HCl, diluted to a definite volume, and the milk-clotting activity determined by our method (2). The activity was found to be 35,000 units per mg. of dry weight before absorption and 9000 units after absorption on crystalline edestin. Variations of this method yielded similar results.

*Experiment 2*—A 10 per cent solution of rennin of the same preparation as used in Experiment 1 was prepared by dissolving in 0.2 per cent HCl and adjusting to pH 6.5 with ammonia. 50 cc. of the filtered solution were cooled to 0° and 1 gm. of crystalline edestin suspended in cold water. The activity was found to be 10,000 units per mg., which was less than half of that of the original preparation.

*Experiment 3*—This experiment differs somewhat from those of Dyckerhoff and Tewes. A quite active rennin preparation, especially furnished by the Chr. Hansen's Laboratory, Inc., was employed. It contained about 90 per cent NaCl as a base. 30 gm. of this rennin were dissolved in 500 cc. of 0.2 per cent HCl, filtered, and adjusted to pH 6.0 with ammonia, and diluted to 600 cc. 60 gm. of crushed hemp-seed were added and extracted at 40° for 30 minutes. Then the extract was filtered and warm water (40°) added until the solution became cloudy. After filtration the filtrate was left for a day at 20°. The globules which precipitated on standing were washed twice with 20 cc. of water and dissolved in 0.2 per cent HCl. The activity of the edestin-rennin globules was 92,000 units per mg. as compared with 400,000 units per mg., the strength of the original material of which 90 per cent was NaCl.

None of these experiments, of which only a few typical ones have been described, indicate an exchange of the protein carrier.

## DISCUSSION

The amazingly rapid and complete digestion of rennin (Tables I and II) by pepsin and trypsin shows that Willstätter's (15) *Träger* theory no longer holds true. Strong evidence is accumulating to indicate that some enzymes at least are proteins, of such a nature, that they may readily be hydrolyzed by proteases of the ordinary type. As we have shown in the above experiments rennin is one of them. Urease we have found to be more resistant (14), and maltase, like rennin, is rapidly digested (16). In the case of maltase we noticed a complete disappearance of the alcohol-precipitable substances. In the case of rennin this could not be tested since the alcohol precipitate of rennin is negligible compared with that of pepsin. Willstätter (17) thinks all enzymes are non-protein substances. According to him the hypothetical carrier, for the existence of which there is very little proof, can be any colloid, which is exchangeable for another colloid. As to the non-protein nature of certain enzyme preparations of Willstätter, it is important to note that some of these findings, as one of us has shown (18), were based on erroneous interpretations. Dry enzyme preparations, containing appreciable amounts of protein, may be diluted to give negative tests for protein and still have powerful enzymic properties. Furthermore, contrary to reports of earlier workers, it was shown that emulsin cannot be completely freed of proteins by the methods which they described, and therefore its protein nature is still a matter for investigation. The categorical assumption, therefore, that all enzymes are of non-protein nature, seems to be baseless.

The digestion of rennin by the other proteases of this group gives us an excellent method for the separation of these two enzymes. It proves that our previous findings were correct. Rennin and pepsin are distinct entities.

That no exchange of the carrier takes place under the conditions described by Dyckerhoff and Tewes is also indicated by our absorption experiments on edestin, since the crystalline protein did not become even half as active as the enzyme preparation with which we started. A statement as to the composition of the enzyme preparations used by Dyckerhoff and Tewes was omitted by them. Commercial enzyme preparations contain "bases" which serve as diluting agents; and consequently such enzymes are not

of high potency. Hence, even if the rennin-edestin crystals were more active than the commercial preparations used by Dyckerhoff and Tewes, it might have meant merely a concentration of the enzyme. Our most active rennin preparation had an activity of over 100 times that of the usual 1 : 30,000 U.S.P. preparations. Since proteins are of amphoteric nature, a chemical combination of the two, the proteose, rennin, and the globulin, edestin, is quite possible. Analytically it is impossible to prove, when impure (crude) rennin solutions are employed for absorption as in the experiments of Dyckerhoff and Tewes, whether in the liquor a measurable decrease in dry weight has taken place, and whether the pure enzyme representing only a fraction of 1 per cent of the crude preparation, has been absorbed, or chemically combined, due to a great affinity to the absorbent. Waldschmidt-Leitz and Kofranyi have used this latter method to show that the protein crystals of Northrop's pepsin are not indispensable and they may be exchanged for plant globulins.

#### SUMMARY

Rennin is rapidly and completely digested by pepsin and trypsin but not by erepsin. In mixtures of rennin and pepsin the latter can easily be separated from the former by this method. These findings support our earlier work on the protein nature of rennin and that rennin and pepsin are distinct entities.

Absorption experiments with rennin on crystalline edestin indicate that in the case of rennin no exchange of the carrier is taking place.

The above facts are strong evidence against the carrier theory.

We are indebted to Dr. J. H. Northrop for the sample of crystalline pepsin and to the Chr. Hansen's Laboratory, Inc., for the especially concentrated rennin.

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# STUDIES ON TRYPSIN

## I. THE CHEMICAL NATURE OF TRYPSIN

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(Received for publication, December 7, 1933)

Recently, Northrop and Kunitz (1) reported the isolation of a crystalline protein from beef pancreas, with constant tryptic and chemical properties, which they contend is identical with the enzyme trypsin. These authors find that crystalline trypsin, besides having the proteolytic powers to be expected, has slight milk-clotting powers which they believe are due to a contamination with a special milk-clotting enzyme of the pancreas.

In 1907, Abderhalden and Pym (2) found that if liver is autolyzed long enough, the filtrates fail to give a biuret test. Because of this we were interested in determining whether pancreatic tissue could be similarly autolyzed until the biuret test was negative and, if so, whether it would still possess enzyme activity, or whether the trypsin would digest itself and thus become inactive.

### *Preparation of Completely Autolyzed Pancreatic Extract*

Pig pancreas was obtained fresh from the slaughter-house, freed from fat, washed, and ground in a meat chopper. To each kilo of ground pancreas, 1000 cc. of 33 per cent ethyl alcohol were added. This was kept in the dark at 20–22°, without shaking, until the biuret test became negative. This was achieved after 18 months. The pH was 6.0. 100 cc. samples of the clear filtrate of the autolyzed extract were placed in cellophane bags and dialyzed against 4 liters of distilled water for 24 hours to remove the autolytic products. After dialysis 3 volumes of acetone were added. A precipitate formed and was centrifuged off. It was redissolved in 20 cc. of distilled water for every 100 cc. of original extract. The water-insoluble part was discarded. This proce-

dure was repeated four times. The final water-soluble precipitate was dried *in vacuo* over  $\text{CaCl}_2$  at  $20^\circ$ .

*General Properties and Analysis of Trypsin from Completely Autolyzed Preparation*

The dry preparation is readily soluble in water and dilute alkali. It is only slightly soluble in dilute acids and is insoluble in organic solvents. The aqueous solution has a reddish brown color. The following protein color and precipitation tests were obtained with a solution containing 10 mg. per cc.: xanthoproteic test, slightly positive; Folin-Denis test, positive; ninhydrin test, positive; biuret test (including the various modifications), negative; Hopkins-Cole test, negative; Millon's test, negative; acetic acid-ferrocyanide test, negative; heat-coagulation test, positive

TABLE I  
*Elementary Composition of Preparation Dried at  $80^\circ$  in Vacuo*

Element	Northrop-Kunitz (1)	Our preparation
	<i>per cent</i>	<i>per cent</i>
Carbon.....	50.00	49.45
Hydrogen .....	7.20	6.24
Nitrogen .....	14.80	11.19
Chloride .....	2.86	0.28
Sulfur.....	1.10	0.25
Phosphorus.....	0.00	0.00
Ash.....	1.20	6.49

only on prolonged boiling, when a slight precipitate was obtained. Nessler's reagent (3) gave no color or precipitate. The sodium nitroprusside test for—SH groups was negative. Saturated solutions of neutral salts gave a precipitate. The isoelectric point is at 6.2. The properties of this substance, considered fully, are not specific for a protein.

The elementary composition of the preparation dried at  $80^\circ$  *in vacuo* is given in Table I. The figures given by Northrop and Kunitz are also shown for comparison.

*Activity of Completely Autolyzed Vacuum-Dried Preparation*

The tryptic activity of the completely autolyzed and vacuum-dried preparation was 22 Willstätter units (4) per 100 mg., when a

6 per cent solution of casein with  $N$   $NH_4Cl$  buffer of pH 8.5 (according to Willstätter) was used as a substrate. Before acetone precipitation and drying this preparation was about 50 per cent more active. The dialyzed solution is many times more active than the undialyzed filtrate of the autolysate. With dialysis, however, there is an enormous loss of active trypsin. In 24 hours more than 80 per cent dialyzed through the cellophane, together with amino acids, etc. The autolytic products inhibit proteolytic activity. Inhibition decreases on dilution of the autolysate. There is also a marked loss of tryptic activity during the course of autolysis. Preparations of much greater activity, but giving a positive biuret test, may be obtained on short autolysis (2 to 3 weeks), followed by short dialysis (16 hours). If pancreatic tissue is autolyzed for about 3 weeks and filtered and the filtrate kept at 20° for a few days, large quantities of tyrosine and cystine crystallize out.

*The rennet activity* of the completely autolyzed and dialyzed trypsin<sup>1</sup> was 1:420 as compared to 1:4,550,000, the strength of our most active rennin preparation (6), when the same  $CaCl_2$ -skim milk of pH 6.2 was used as a substrate. This is about 0.0001 the milk-coagulating power of rennin. That even this slight milk-clotting power is a function of the trypsin itself is shown in another paper (5).

#### DISCUSSION

Trypsin, as obtained by complete autolysis (18 months), dialysis, and precipitation by acetone, does not resemble a protein of the ordinary type. The activity of our preparation is about the same as that of crystalline trypsin (1). We have obtained, however, more active preparations on short autolysis, which gave protein color tests. The protein-free preparation has milk-clotting power which, however, is less than that of those giving protein color tests. It appears that either trypsin is not a protein as Willstätter and Rohdewald (7) indicate in their recent paper, or that split-products of the protein, trypsin (Northrop-Kunitz), retain a great part of its activity, even after complete autolysis. Willstätter and Rohdewald do not give a very complete description of the chemical

<sup>1</sup> The trypsin solution was diluted to contain approximately 1 mg. per cc. (5).

nature of their trypsin preparation, apparently because of their belief that their substance may only be a carrier of the active trypsin (see p. 79 of their paper).

“Das Enzym, aus einer aktiven Gruppe und dem notwendigen kolloiden Träger noch unbekannter Art bestehend, kann mit verschiedenen anderen hochmolekularen Stoffen assoziiert, z. B. zusammen mit Proteinen in Form von festen Lösungen oder von Adsorptionsverbindungen auftreten, oder sein kolloider Träger kann mit weiteren Eiweissgruppen oder anderen hochmolekularen Stoffen chemisch verbunden sein.”

It seems to us that even if certain enzymes are typical proteins, as we have shown in the case of rennin (6, 8), there is a possibility of obtaining others in a protein-free state.

The effect of trypsin on casein is described in a separate paper (5).

#### SUMMARY

A protein-free trypsin preparation has been obtained.

18 months of autolysis of pancreatic tissue does not result in the destruction of the proteolytic or milk-clotting power of trypsin.

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## STUDIES ON TRYPSIN

### II. THE EFFECT OF TRYPSIN ON CASEIN

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It has long been the opinion of biochemists that all proteases have the power of clotting milk as a result of a slight cleavage of the casein molecule. Recently, however, Northrop and Kunitz (1) have thrown some doubt upon this general opinion as regards trypsin. They found that purified trypsin loses a great part of its milk-clotting power during the course of its purification. They, therefore, conclude that the slight milk-clotting action of their trypsin is due to a contamination of trypsin with a special milk-coagulating enzyme. The following experiments were performed to throw light on this subject.

#### *Experiments Showing That Milk-Clotting Power Is a Function of Trypsin Molecule, and That Trypsin Can Clot Milk Only within Certain Range of Concentration*

Table I shows that concentrated trypsin solutions do not clot milk, whereas dilute solutions do, up to a limited dilution. The experiments of Table II show that after the casein has been exposed to concentrated solutions of trypsin (0.6 to 4 per cent) for a short time (10 minutes) no clot occurs. If rennin is subsequently added to this solution, no clot can be obtained since the trypsin has completely changed the casein molecule or complex. Therefore, Ca-paracaseinate cannot form.

Experiments incorporated in Table III show that concentrated solutions of trypsin can clot milk if the pH is adjusted to the acid side so that the action of trypsin is depressed. It is seen that between pH 6.1 and 5.6 trypsin clots milk readily but does not between pH 6.4 and 6.8. The milk-clotting power of rennin and

## Effect of Trypsin on Casein

TABLE I  
*Showing That Trypsin Can Clot Milk Only within a Certain Range of Concentration*

Trypsin, per cent. . . . .	2.00	1.00	0.75	0.50	0.20
Results after 10 min. . . . .	No clot	No clot	No clot	Slight clot	Slight clot

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Trypsin, per cent. . . . .	0.10	0.08	0.05	0.03	Remarks
Results after 10 min. . . . .	Slight clot	Heavy curd	Heavy curd	No clot	After 5 hrs. results remained same

To 10 cc. samples of  $\text{CaCl}_2$ -milk, 1 cc. of each of the various trypsin solutions was added. The preparation of milk was the same as of that described in an earlier paper (2) except that Klim was used instead of skim milk. The pH of this milk was 6.4, as determined by the electrometric method. Temperature  $40^\circ$ .

TABLE II  
*Showing Rapid Effect of Trypsin on Casein*

Experiment No.	Per cent of trypsin solution	Results after 10 min.	After 10 min. tryptic digestion 1 cc. rennin solution containing 13,000 units* per cc. was added	Remarks
1	0.015	No clot	Clot in 8 min.	Trypsin too dilute to affect casein molecule (or complex)
2	0.030	" "	" " 7 "	
3	0.300	Clot	No rennin added	At this pH (6.4) and concentration trypsin readily clots milk
4	0.600	No clot	No clot in 5 hrs.	Trypsin solutions of these concentrations change casein to such degree that insoluble Ca-paracaseinate cannot form, even on addition of active rennin
5	1.000	" "	" " " 5 "	
6	4.000	" "	" " " 5 "	

To 10 cc. of  $\text{CaCl}_2$ -milk (Klim), of pH 6.4, 1 cc. of the trypsin solution was added. Temperature  $40^\circ$ .

\* A unit is the amount of milk (pH 6.2) in mg. clotted by 1 mg. of rennin (3, 4).

pepsin, as shown in Table III, increases with the increase in H ion concentration. At pH 6.8 the rennet activity of rennin is very small and the rennet activity of pepsin appears to be nil. The pepsin was Parke, Davis and Company's, 1:3000. The rennin was the same as that used in a preceding paper (3). The trypsin was an aqueous solution of Fairchild Brothers and Foster, of which only a very small part was water-insoluble. The tryptic activity of this aqueous solution was 4.5 Willstätter (5) units per 100 mg.

TABLE III  
*Milk-Clotting Activity of Rennin, Pepsin, and Trypsin at Various pH*

pH	Clotting time of rennin	Clotting time of pepsin	Clotting time of trypsin
	min.	min.	min.
5.6	3.5	1.5	3.0
5.9	4.5	2.0	4.0
6.1	8 0	3.0	6.0
6.4	10 0	11.0	No clot in 40 min.*
6.6	20.0 very slight	No clot in 40 min.	" " " 40 " *
6.8	36.0 " "	" " " 40 "	" " " 40 " *

The pH of the milk was adjusted with HCl or NaOH and electrometrically controlled. To 10 cc. samples of milk, 0.5 cc. of each of the enzyme solutions was added. The trypsin solution was 1 per cent. The pepsin solution contained 18,000 rennet units per cc. The rennin solution had an activity of 16,000 units per cc. Temperature 40°.

\* No clot occurred after adjustment to pH 5.6.

The protein-free trypsin described in the preceding paper (2) was used in similar experiments, with corroborative results.

#### DISCUSSION

The milk-coagulating power of trypsin is restricted to certain low concentrations. When higher concentrations of trypsin are used, the cleavage of the casein molecule is so rapid that apparently the paracasein stage is reached and passed without the formation of Ca-paracaseinate. This is shown by the fact that no Ca-paracaseinate forms upon subsequent addition of rennin to such a tryptically digested milk.

If high concentrations of trypsin are employed, milk clotting



may be brought about if the pH of the milk is adjusted to an unfavorable degree. In this way proteolytic activity is inhibited and digestion proceeds so slowly that the paracasein stage is not passed before Ca-paracaseinate can be formed.

The velocity of milk clotting is in general proportional to the  $C_H$  of the milk in the case of all three enzymes, rennin, pepsin, and trypsin. The higher the H ion concentration, the quicker the curd formation.

#### SUMMARY

Trypsin can clot milk only within a certain limited range of concentration; if too concentrated or too dilute no clotting will occur. If, however, the milk is so acid that the proteolytic activity of the trypsin is depressed, clotting may occur; otherwise trypsin changes the casein molecule very rapidly beyond the paracasein stage and the casein will not clot even after subsequent addition of a very active rennin solution. That the milk-coagulating power is a function of the trypsin molecule can be clearly seen from these experiments.

The velocity of milk coagulation is proportional to the H ion concentration of the milk in the cases of rennin, pepsin, or trypsin.

We wish to thank Mr. D. Mishkind for technical assistance.

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# **CALCIUM CONTENT OF THE AQUEOUS AND VITREOUS HUMORS AND SERUM**

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(Received for publication, October 14, 1933)

Lebermann (1) was the first to publish comparative data on the calcium content of the aqueous and vitreous humors from identical animals. He found the calcium content of the aqueous humor of ox eyes to be considerably higher than that of the vitreous humor, the averages being 10.1 and 8.1 mg. per 100 gm. respectively. In 1926 Tron (2) determined the calcium content in the aqueous and vitreous humors and the serum of oxen and horses. His average values, expressed in mg. per 100 gm., are as follows: oxen, 6.2 for aqueous humor, 6.9 for vitreous humor, and 10.3 for serum; horses, 7.4, 8.2, and 11.3 respectively. In 1929 O'Brien and Salit (3) carried out similar investigations on the aqueous and vitreous humors of oxen. Their results, however, differ widely from those of Lebermann, but less widely from those of Tron, in that the aqueous humor apparently contains considerably less calcium than does the vitreous humor. The average calcium values, according to these investigators, are 5.17 mg. per 100 cc. for aqueous and 7.20 mg. for vitreous humor. The differences in results may be due to differences in technique. Lebermann employed the gravimetric method for estimation of the quantities of fluids; extremely small amounts (0.25 gm.) were used for each determination and as a result the possibility of experimental error was great. Tron used the same method but employed larger quantities (0.7 to 1.0 gm.). There are two procedures in the gravimetric method: (1) The weight of the fluid is determined by the weight of the syringe and fluid minus the weight of the syringe. In this manner no loss in weight, due to evaporation, can occur during weighing. (2) The fluid is discharged on a pad of filter

paper of known weight and rapidly weighed. This technique has been employed widely in European countries, especially when working with blood. If one uses small quantities of fluids, it is evident that concentration of the fluids, due to evaporation, is unavoidable. Unfortunately the above authors do not state which of the two procedures was employed. If the second procedure was employed, it is probable that the protein-free aqueous humor lost water more rapidly than the vitreous humor, since the latter contains hydrophilic proteins. In our experiments of 1929 the aqueous humor was never exposed to air; it was kept in a well oiled graduated syringe until measured out, either directly from the syringe or with a volumetric pipette, after its discharge under oil into a small test-tube. If as much as 2 cc. of aqueous humor were obtained from a single eye, the calcium was determined on this amount; however, in most analyses the aqueous humor was pooled and 4 cc. were used in each determination. The vitreous humor, after rapid removal from the eye, was immediately covered with oil and liquefied under this medium with a special instrument. The necessary amount, 4 cc. in each determination, was then removed with a volumetric pipette.

Quite recently, Winternitz and Stry (4) published the results of investigations of the calcium content of the aqueous humor, vitreous humor, and serum of horses. They, like Lebermann, found the calcium of the aqueous humor higher than that of the vitreous humor; *i.e.*, 7.0 mg. per cent for aqueous humor, 6.4 mg. per cent for vitreous humor, and 12.1 mg. per cent for serum. It is not stated whether the gravimetric or the volumetric method was used for ascertaining the quantities of fluids, neither is there a note of the amounts used in each determination.

In view of the disagreement between the results of the above authors and those published by O'Brien and Salit, this study has been repeated on the aqueous humor, vitreous humor, and serum of oxen, by the Kramer-Tisdall (5) method. This method appears to be most widely used, and was employed by Winternitz and Stry, as well as by O'Brien and Salit. Lebermann determined the calcium acidometrically by his own method (6) and Tron used the de Waard method (7). In the present study separate determinations of calcium content were made on the aqueous humor, vitreous humor, and serum in calves, young adult cattle, and aged

cattle. In calves the combined aqueous humor (1.0 to 3.3 cc.) of both eyes was used in each analysis. In young adult and old cattle most of the determinations were made on the aqueous humor of each eye, with quantities varying between 1.0 and 2.7 cc. A few analyses were carried out on the pooled aqueous humor of both eyes, in which instance the quantities varied from 1.5 to 3.0 cc. In the analyses of vitreous humor and serum, 4 cc. of each fluid were used. In other details, the technique formerly used by O'Brien and Salit was followed.

The results obtained on the aqueous and vitreous humors are essentially the same as those published by O'Brien and Salit in 1929. Only in a single instance does the calcium content of the aqueous humor of both eyes exceed that of the vitreous humor (Table I, 1 to 2 years old). In the present study, however, an effort was also made to determine the calcium distribution with respect to age. Only approximate ages could be obtained, and the animals were roughly classed into three groups: young calves, 4 to 8 weeks old; young adults, 1 to 2 years old; and older animals, 5 to 10 years old.

It is apparent, from Tables I and II, that young calves have the highest calcium concentration in all three fluids; *i.e.*, aqueous humor, vitreous humor, and serum, the respective average values being 5.82 mg., 8.34 mg., and 11.54 mg. of calcium per 100 cc. of fluid. The average values for cattle between the ages of 1 and 2 years are as follows: aqueous humor 5.42 mg., vitreous humor 7.17 mg., and serum 10.32 mg.; the averages for older cattle are: 4.60 mg., 6.75 mg., and 9.69 mg. respectively.

Data on aqueous and vitreous humors, listed in Table I, are based on strictly fresh fluids; *i.e.*, fluids which were obtained within 3 to 5 minutes after death of the animal. In several instances, however, the enucleated eyes were purposely allowed to stand for varying periods of time before removing the fluids (Table II). In such cases it was found that the calcium content of the aqueous humor had increased, in some instances reaching or even surpassing that of the vitreous humor. This increase in calcium of the aqueous humor probably was due to diffusion from the vitreous humor. At the present time there is no way of ascertaining the precise nature of this process; one can think only of certain possibilities: (1) Since the calcium content of the vitreous

TABLE I  
Minimum, Maximum, and Average Calcium Values of Fresh Aqueous Humor, Vitreous Humor, and Serum\*

Animal	Age	No. of animals	Aqueous humor		Vitreous humor		Serum
			cc.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Calves.....	4-8 wks.	4	1.0-2.7	5.01-6.14	7.47-9.82	10.61-12.66	
Averages.....			1.95 (8)	5.82 (8)	8.34 (8)	11.54 (4)	
Young adult cattle.....	1-2 yrs.	30	1.0-3.0	3.64-8.60	6.10-8.16	9.04-12.12	
Averages.....			1.46 (60)	5.42 (60)	7.17 (60)	10.32 (15)	
Aged cattle.....	Above 5 yrs.	16	1.5-4.1	3.27-5.85	6.03-8.33	8.43-11.23	
Averages.....			2.09 (30)	4.60 (29)	6.75 (32)	9.69 (8)	

TABLE II  
Minimum, Maximum, and Average Calcium Values of Aqueous and Vitreous Humors of Not Strictly Fresh Eyes\*

Animal	Age	No. of animals	Aqueous humor		Vitreous humor		Hrs. after death
			cc.	mg. per cent	mg. per cent	mg. per cent	
Calves.....	4-8 wks.	3	1.6-3.3	6.96-8.84	6.93-7.53	4-11	
Averages.....			2.45 (4)	7.90 (2)	7.23 (3)	8.7	
Young adult cattle.....	1-2 yrs.	11	1.0-1.7	4.42-7.26	6.49-8.18	0.5-10.0	
Averages.....			1.36 (21)	6.14 (21)	7.30 (21)	2.3	
Aged cattle.....	Above 5 yrs.	9	1.53-2.10	4.82-6.96	6.23-7.33	2-5	
Averages.....			1.24 (18)	6.03 (18)	6.74 (18)	3.0	

\* Figures in parentheses indicate the number of eyes used or the number of analyses included in the averages.

humor exceeds that of the aqueous humor by approximately 32.3 per cent in young adult cattle, this calcium may be considered as being in combination with the protein of the vitreous humor. After death the calcium proteinate may disintegrate and release the calcium, which then readily passes into the aqueous humor. (2) O'Brien and Salit (3) have shown that, when the eyes are allowed to stand for some time after death of the animals, the protein content of the aqueous humor increases enormously. This suggests that the living membrane, which is evidently semipermeable, may become more permeable or porous after death. If such is the case, even in the absence of further dissociation of calcium proteinate, the latter finds no difficulty in passing through the altered membrane.

#### SUMMARY

Comparative studies were made on the calcium content in the aqueous humor, vitreous humor, and serum of cattle. The average values in terms of mg. of calcium per 100 gm. of fluid were as follows:

	Calves	Young adult cattle	Aged cattle
Aqueous humor.....	5.82	5.42	4.60
Vitreous " .....	8.34	7.17	6.75
Serum.....	11.54	10.32	9.69

As indicated above, calcium is present in the greatest concentration in calves and gradually decreases with age. The average calcium content of the aqueous humor, vitreous humor, and serum of calves exceeds that of young adult cattle by 8.5, 16.3, and 11.8 per cent respectively; that of young adult cattle exceeds that of aged cattle by 18.0, 6.2, and 6.5 per cent respectively. The calcium content of the vitreous humor exceeds that of the aqueous humor in 4 to 8 week-old calves by 43.4 per cent, in 1 to 2 year-old cattle by 32.3 per cent, and in 5 to 10 year-old cattle by 46.7 per cent. However, if the eyes are enucleated and allowed to stand for a few hours, the calcium content of the aqueous humor invariably increases, approaching or even surpassing that of the vitreous humor. The ratios between the calcium content of aque-

ous humor and serum in the three age groups are as follows: calves, 1:1.98; young adult cattle, 1:1.90; older cattle, 1:2.11. The respective ratios between the calcium content of vitreous humor and serum are 1:1.39, 1:1.44, and 1:1.43. There is some consistency with respect to the ratios between aqueous humor and serum and more consistency with respect to the ratios between vitreous humor and serum. These ratios indicate that the calcium content of the ocular humors is directly dependent on the calcium content of the serum.

Dr. C. S. O'Brien, Head of the Department of Ophthalmology, gave valuable help throughout this work.

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## GLYOXALASE

### I. THE APPLICABILITY OF THE MANOMETRIC METHOD TO THE STUDY OF GLYOXALASE

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Since the discovery (1, 2) of the existence in cells of the enzyme glyoxalase, which converts methylglyoxal into lactic acid, and of the ability of many types of cells to produce methylglyoxal from sugars under proper conditions, it has generally been assumed that glyoxalase and methylglyoxal occupy key positions in the scheme of cellular carbohydrate metabolism. Recent investigations of Embden and coworkers (3) and Meyerhof (4) have cast some doubt on this hypothesis. According to these authors, muscle extracts metabolize carbohydrate to lactic acid essentially as follows: hexosediphosphate  $\rightarrow$  triosemonophosphate  $\rightarrow$  phosphoglyceric acid  $\rightarrow$  pyruvic acid  $\rightarrow$  lactic acid. There is no place in this scheme for methylglyoxal or glyoxalase. Barrenscheen and Beneschovsky (5), on the other hand, have reported that liver, brain, and red blood cells, in contrast to muscle, cannot convert added phosphoglyceric acid into pyruvic acid. They suggest the possibility that several distinct mechanisms may be involved, the type depending on the particular tissue in question. Dickens (6) found that the concentration of iodoacetic acid necessary to inhibit the action of glyoxalase in intact tissues is of the same order as that required to inhibit the entire glycolysis process, and he sees, in this, added support for the belief that methylglyoxal and glyoxalase play a part in carbohydrate breakdown. The discovery by Lohmann (7) that glutathione is the natural activator of glyoxalase is also of interest. It seems reasonable, therefore, to attach an important physiological significance to the wide-spread occurrence in all types of cells of these two closely related substances, glutathione and glyoxalase.



We have undertaken a study of the enzyme glyoxalase with the view of obtaining additional evidence as to its true function, and its possible relation to the high glycolytic activity of the cancer cell. In line with the general program of this laboratory of determining the quantitative distribution of enzymes in cancer and normal tissues, we are carrying out similar studies on glyoxalase.

The present paper deals with the applicability of manometric methods to the study of glyoxalase, and with the conditions which must be observed in the quantitative determination of enzyme activity. In principle, the method depends on the measurement of the volume of carbon dioxide produced in a given period of time as a result of the neutralization, by sodium bicarbonate, of the lactic acid formed. Lohmann was the first to use manometric methods extensively with this enzyme, previous workers generally having employed chemical methods for determining the amount of lactic acid formed, or of methylglyoxal disappearing. In fact, it has been claimed (8) that manometric methods are not suitable because of the errors introduced by gas formation in side reactions between the substrate and proteins present in the enzyme solution. We have found that, under proper conditions, this blank gas formation is insignificant, or may be easily corrected. The manometric method possesses certain obvious advantages over the chemical method, for example, in the saving of time and labor. At the same time, it provides an independent method of checking the results obtained by the chemical method.

The work here reported has been carried out entirely with acetone-yeast glyoxalase. Experiments thus far completed with glyoxalase in animal tissue indicate an entirely analogous behavior, as far as the kinetics of the reaction are concerned. The effect of various factors on the rate of the enzyme reaction has been investigated: manipulation, temperature, glutathione concentration, methylglyoxal concentration, and enzyme concentration. These studies have enabled us to define the conditions under which proportionality exists between the quantity of enzyme and the rate of reaction, and have led to the conclusion that the method is applicable to the accurate determination of the relative activities of different enzyme solutions.

The best conditions for determining the activity of the enzyme were found to exist at 25°, and in the presence of relatively low

glutathione and methylglyoxal concentrations. Under these conditions the rate of reaction is constant, independent of the methylglyoxal concentration, and directly proportional to the amount of enzyme suspension used. At 37°, the rate decreases with time almost from the start of the experiment because of enzyme destruction. The same decrease in rate is also observed at 25° when high concentrations of glutathione are used, due perhaps to a slight toxic effect. Furthermore, in this case the rate increases considerably as the methylglyoxal concentration is increased, and proportionality between amount of enzyme and activity no longer holds. The rate of reaction is dependent on the concentration of glutathione, increasing rapidly to a constant value and finally decreasing slightly as the concentration of glutathione is increased.

A few experiments were carried out with solutions of pure glutathione and methylglyoxal which indicate, in agreement with the views of Jowett and Quastel (9), that compound formation occurs between these two substances, the complex thus formed being the true substrate for the enzyme. The reaction proceeds quite rapidly at room temperature, but appears to reach an equilibrium.

It was found that iodoacetic acid inhibits acetone-yeast glyoxalase by destroying the glutathione; the enzyme itself is not damaged by this reagent. This agrees with the findings of Dickens for glyoxalase in rat liver.

#### EXPERIMENTAL

*Materials*—Acetone-yeast was prepared from fresh pressed bakers' yeast (Fleischmann) according to the procedure of Albert, Buchner, and Rapp (10). In the dry state this material is stable for long periods of time, and provides an easily available, constant source of glyoxalase. It possesses the added advantage that the glutathione present may be completely removed by simply washing with water. For the preparation of washed yeast, 1 gm. of acetone-yeast was suspended in 50 cc. of distilled water and centrifuged. The liquid was decanted and the washing repeated. The solid residue was then made up into a 5 or 10 per cent suspension and kept in the ice box. This washed material is inactive towards methylglyoxal, but becomes active upon addition of glutathione.

Methylglyoxal was prepared by distillation of a solution of dihydroxyacetone in dilute sulfuric acid according to the method

of Neuberg and coworkers (11). The exact concentration of the methylglyoxal in the distillate was determined by heating 1 cc. with 10 cc. of  $N$  NaOH for 5 minutes in a boiling water bath and estimating the lactic acid formed by the method of Friedemann, Cotonio, and Shaffer (12). In this paper amounts of methylglyoxal are expressed in terms of their lactic acid equivalents.

Glutathione used in these experiments was the crystalline product obtained from the Eastman Kodak Company. Standard solutions prepared by weighing gave the theoretical titer by the iodate procedure of Woodward and Fry (13). By this method glutathione values are obtained for freshly prepared suspensions of acetone-yeast which are only slightly higher than indicated by the glyoxalase activity. But in suspensions which have stood for long periods of time, autolysis causes the formation of substances other than glutathione which take up iodine.

*Manipulation*—Determinations of enzyme activity were made in the simple Warburg manometric apparatus, the manometer cups having a single side bulb and a total volume of about 18 cc. The procedure described by Lohmann (7) was in general closely followed.

This consists in placing into the cups the desired quantities of enzyme suspension, methylglyoxal, and glutathione, together with 0.4 cc. of sodium bicarbonate (0.2  $M$ ) and sufficient water to make a total volume of 2 cc. Depending on the factor being studied, one or more of the constituents may be placed in the side bulb and the rest in the main chamber. The cups are then attached to the manometers, shaken at constant speed in a constant temperature bath, and saturated with  $CO_2$  by means of a rapid stream of 5 per cent  $CO_2$  in nitrogen. The contents of the side bulb are then tipped into the main chamber. The manometer stop-cock is kept open for 2 minutes after tipping (the so called open period), the manometer liquid leveled, and the stop-cock closed. The manometer reading multiplied by the vessel constant gives the number of c.mm. of  $CO_2$  formed in any given time by neutralization of the lactic acid produced. Since our interest was only in the rate of the reaction, and not in the total amount of lactic acid formed, it was unnecessary to apply the  $CO_2$  retention correction, which is quite small in any case. Lohmann has shown that, when this factor is applied, the chemical and manometric methods for total lactic acid formed agree within a few per cent.

It makes little difference which constituent is placed in the side bulb, although, as a rule, slightly higher  $\text{CO}_2$  values are obtained with methylglyoxal or methylglyoxal plus glutathione than with yeast or glutathione alone. A uniform procedure should be used, therefore, in any series of comparative experiments.

In agreement with the observation of Jowett and Quastel, it was found that immediately after tipping the contents of the side bulb into the main chamber, a rapid evolution of gas occurs which is not a true measure of glyoxalase activity. It is, therefore, necessary to open the manometer stop-cock for a short time to permit a constant rate to become established. Numerous experiments have shown that 2 minutes is sufficient and this time was adopted in all our work.

In view of the previously mentioned objection to the manometric method for glyoxalase study, a detailed investigation was made of the blank gas evolution produced by side reactions. It has been shown (9) that glutathione and methylglyoxal react in the presence of bicarbonate, resulting in the formation of a small quantity of gas. We have found that when the 2 minute open period is observed no gas formation can be detected in mixtures of glutathione, methylglyoxal, and bicarbonate, at least within the limits of concentration usually employed in our experiments. As is shown in a later section of this paper, when the concentration of methylglyoxal is greatly in excess of that of glutathione, the reaction between these two substances is so rapid that it is completed before the end of the open period and no gas evolution is obtained. The open period also allows for the neutralization of the glutathione and of any acids present in the methylglyoxal.

Any blank gas evolution which occurs is therefore due to other factors besides glutathione, probably to reactions between amino compounds present in the yeast and methylglyoxal (14). The size of the blanks to be expected was determined with washed (glutathione-free) yeast, and also with unwashed yeast which had been heated to  $75^\circ$  for 30 minutes to destroy the enzyme. With 2 mg. of methylglyoxal and 0.4 cc. of bicarbonate, the volumes of blank  $\text{CO}_2$  obtained in 20 minutes ranged from 2 to 11 c.mm. as the amount of yeast was increased from 10 to 50 mg., the range within which most of our work was done. The error introduced by this blank will depend naturally on the activity of the particular reaction mixture being studied, but in general will be between 1 and 5 per cent.

For many types of experiments this is no serious objection. In others, it may easily be corrected for in a blank determination with washed yeast. It was found that most of the blank gas evolution occurs within the first 5 minutes, and that, after 10 minutes, only negligible amounts are formed. Therefore, whenever possible, the procedure was adopted of measuring the enzyme suspension and the methylglyoxal into the main chamber of the manometer cup as the first step in the preparation of the reaction mixtures. The reaction between the methylglyoxal and substances present in the yeast then takes place while the rest of the additions are being made and while the  $\text{CO}_2$  saturation is carried out. Since this usually requires about 10 minutes, the blank is reduced to such small proportions that it may be entirely neglected.

The results which have been obtained by the manometric method are reproducible within 2 per cent. This is probably close to the limit of accuracy to be expected in the measurement of small volumes of yeast suspensions.

*Stability of Acetone-Yeast Glyoxalase*—Acetone-yeast retains its original glyoxalase activity for several months when kept dry and cold. Aqueous suspensions are much less stable. Table I shows the rate at which the activities of 10 per cent suspensions of washed and unwashed yeast decrease when allowed to stand at  $0^\circ$  and  $37^\circ$ . The activities (c.mm. of  $\text{CO}_2$  in 20 minutes) were measured at intervals in reaction mixtures containing 0.4 cc. of yeast suspension, 0.4 cc. of bicarbonate, and 2 mg. of methylglyoxal. To the unwashed yeast, no glutathione was added, while to the washed suspensions, 0.25 mg. per manometer wt. was added. This amount of glutathione, when added to 0.4 cc. of the freshly prepared washed yeast, gave practically the same activity as was obtained with 0.4 cc. of the fresh unwashed suspension. The latter, by titration, contained 0.32 mg. of glutathione, the difference undoubtedly being due to other titratable material. The temperature at which the activities were measured was  $25^\circ$ .

At  $0^\circ$ , the suspension of washed yeast retains practically its original glyoxalase activity for 24 hours, while, at  $37^\circ$ , it has become almost inactive. Unwashed suspensions, on the other hand, become inactive in 24 hours both at  $0^\circ$  and at  $37^\circ$ . The figures in parentheses show the activities produced by adding 0.25 mg. of glutathione to 0.4 cc. of these suspensions. It is clear that the de-

crease in activity which occurred in 24 hours at 0° was due simply to destruction of glutathione. The 37° suspension, however, cannot be reactivated with glutathione, the enzyme having been destroyed as well.

Suspensions of washed yeast kept at 0° for 24 hours take up no iodine; the nitroprusside reaction likewise remains negative. At 37°, however, the iodine uptake increases considerably with time, and a strong nitroprusside reaction develops. Since the glyoxalase activity without added glutathione remains zero, this must be accounted for by the formation, by proteolysis, of substances other than glutathione which take up iodine and give the nitroprusside test. In unwashed yeast, likewise, the iodine uptake and the

TABLE I

*Stability of Acetone-Yeast Glyoxalase (C.Mm. CO<sub>2</sub> Evolved in 20 Minutes)*

Time of standing  <i>hrs.</i>	Washed + GSH		Unwashed	
	0°	37°	0°	37°
0	165	165	167	167
1	171	162	143	141
5	168	113		
24	161	29	25 (155)	33 (37)
48	116			

The figures in parentheses show the activities produced by adding 0.25 mg. of GSH at 37°.

intensity of the nitroprusside reaction increase with time, despite the fact that the glyoxalase activity decreases.

*Rate of Glyoxalase Action*—In our early experiments a temperature of 37° was employed, since that is the temperature at which most of the chemical work reported in the literature has been done. It was found that the reaction rate is not constant here, but begins to decrease almost from the start. Similar results have been reported by Kuhn and Heckscher (15) for pig liver glyoxalase at 30° and by Lohmann (7) for air-dried yeast at 28°. The latter found, however, that with rat liver glyoxalase and muscle glyoxalase constant rates were obtained until all the methylglyoxal had been converted into lactic acid. The decrease in rate observed in

our experiments at 37° is not due to inhibition by the reaction product, nor to the increasing acidity, as could be shown by addition of lactic acid to the mixtures at the start. No difference in rate could be observed in the presence of the added lactic acid. The calculated constants for a monomolecular reaction decreased with time, as was also found by Kuhn and Heckscher. It appears probable that destruction of enzyme is responsible for the decrease in rate, a result which might be expected on the basis of the stability experiments.

As shown in Fig. 1, at 25° the reaction rate is constant until practically all the methylglyoxal has been used up. These results were obtained with the amounts of washed yeast indicated, together with 1 mg. of methylglyoxal, 0.4 cc. of bicarbonate, and 0.25 mg. of glutathione (side bulb), in a total volume of 2 cc. Although the CO<sub>2</sub> values have not been corrected for retention, the total obtained corresponds to 96 to 98 per cent conversion of the methylglyoxal to lactic acid. This indicates that only a very small proportion of the methylglyoxal has been destroyed by interaction with protein.

It must be pointed out that one does not obtain a constant rate under all conditions, even at 25°. For example, if the reaction runs for more than an hour, the rate begins to fall off, probably because of enzyme destruction. This is no serious objection since most experiments are limited to 20 minutes. Furthermore, when the concentrations of glutathione and methylglyoxal are high (above 1.0 and 5.0 mg. respectively) toxic effects appear which tend to decrease the rate.

These results, as well as those of the preceding section, demonstrate the inadvisability of carrying out long digestions with glyoxalase at 37°, as has frequently been done in the past.

*Glutathione Concentration and Activity*—Fig. 2 shows the effect of increasing the concentration of glutathione on the glyoxalase activity of acetone-yeast. The reaction mixtures contained 0.2 cc. of a 5 per cent suspension of unwashed yeast (containing 0.075 mg. of glutathione by iodate titration), 0.4 cc. of bicarbonate, 2.0 mg. of methylglyoxal, and increasing amounts of glutathione, in a total volume of 2 cc. Methylglyoxal and glutathione were placed in the side bulb. The determinations were carried out at 37°, and the values obtained corrected for the yeast blank. The same type of curve was obtained also at 25°.

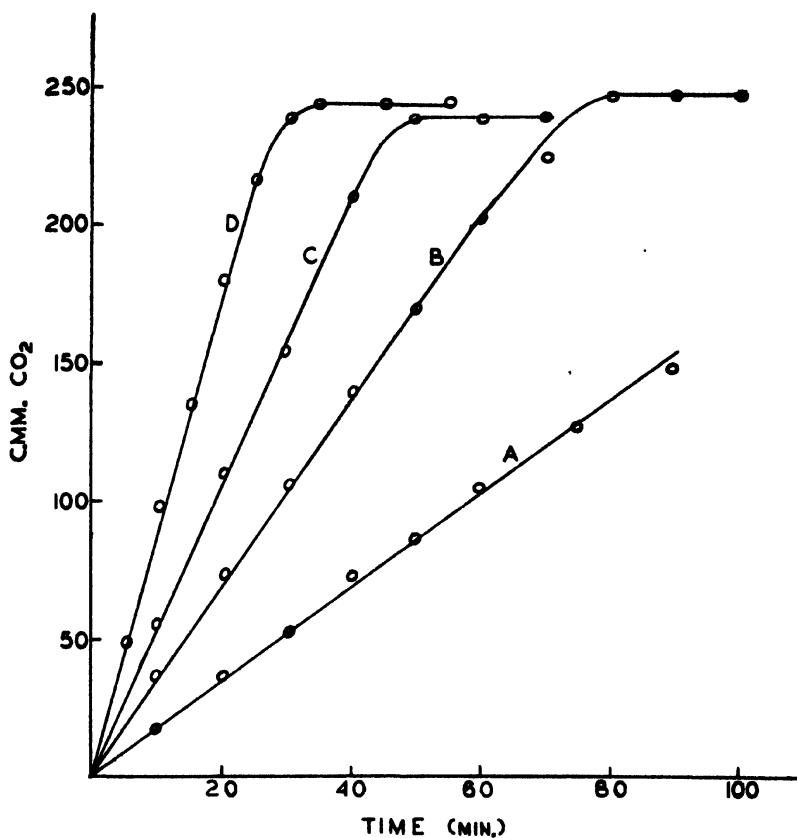


FIG. 1. Rate of glyoxalase action at 25°. Mixtures A, B, C, and D contained 10, 20, 30, and 50 mg., respectively, of washed acetone-yeast in a total volume of 2 cc.

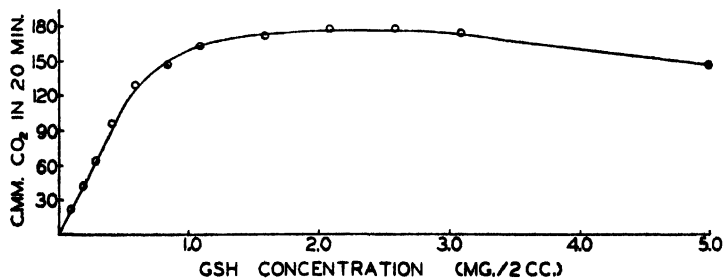


FIG. 2. Effect of glutathione concentration on glyoxalase activity



The activity increases rapidly, reaches a practically constant value, and finally decreases slightly, as the glutathione concentration is increased from 0 to 5 mg. per reaction vessel. The decrease in activity in the higher concentrations is not due to increased acidity produced by the large quantity of glutathione present, similar results being obtained when the latter is previously neutralized with NaOH. It is probably due to a slight toxic effect. In the region of low glutathione concentrations, doubling the latter increases the reaction rate 1.8 times, as compared to a value of 1.7 found by Lohmann for rat liver glyoxalase.

The maximum in the activity curve obtained with 2 mg. of glutathione may be said to represent the full activity of the enzyme. However, it represents the full activity only for the particular concentration of methylglyoxal being used. For example, if the amount of glutathione is held constant at 2 mg., and the quantity of methylglyoxal is increased to 4 and 8 mg., the amount of CO<sub>2</sub> evolved in 20 minutes is increased to 308 and 448 c.mm. respectively, as compared to 180 c.mm. with 2 mg. of methylglyoxal. Apparently the activity produced by this amount of glutathione is dependent on the methylglyoxal concentration as well, and for every concentration of the latter a different glutathione-activity curve exists. Lohmann obtained maximum activation of rat liver glyoxalase with considerably lower glutathione concentrations than was the case in our experiments. This is probably due in part at least to the fact that he used smaller quantities of methylglyoxal.

Much of the work done on this enzyme in the past is of little value because no account has been taken of the varying amounts of glutathione which are extracted from the tissues along with the enzyme, or of the rapid destruction of this glutathione which occurs on standing, with consequent changes in the activity of the enzyme.

*Methylglyoxal Concentration and Activity*—As is clear from Fig. 3, the effect on glyoxalase activity of increasing the methylglyoxal concentration is dependent on the concentration of glutathione present. In these experiments, which were carried out at 25°, the reaction mixture contained 0.4 cc. of 5 per cent washed yeast, 0.4 cc. of bicarbonate, and the indicated amounts of methylglyoxal and glutathione, the latter being placed in the side bulb.

In the presence of low glutathione concentrations, the glyoxalase

activity is independent of the methylglyoxal concentration within the range from 1.0 to 5.0 mg. With high glutathione, on the other hand, the activity is greatly influenced by the amount of methylglyoxal present. With intermediate glutathione concentrations,

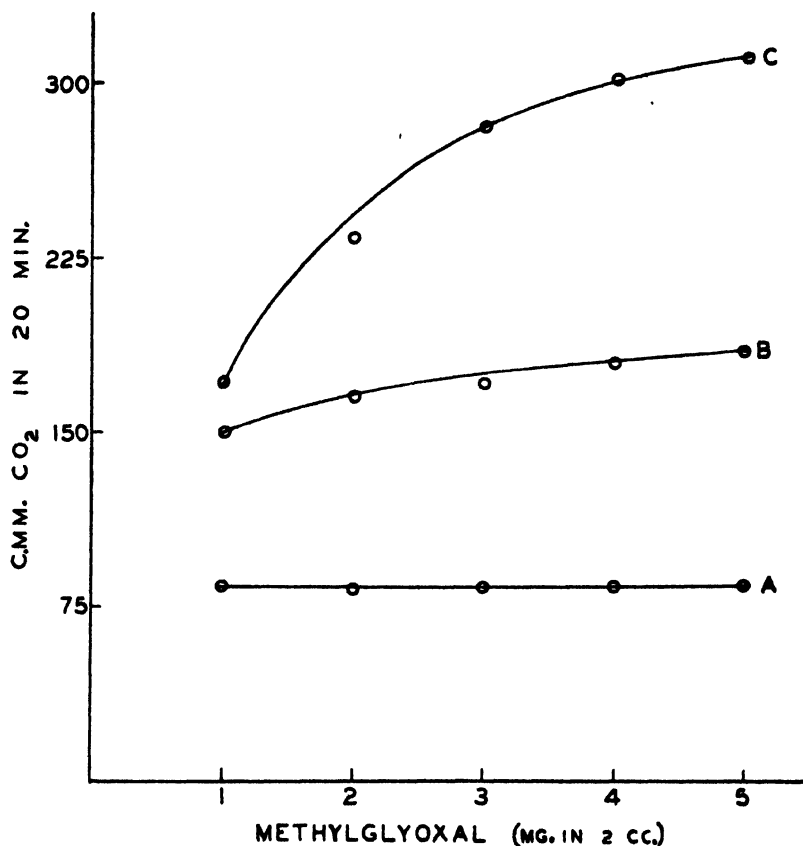


FIG. 3. Effect of methylglyoxal concentration on glyoxalase activity. Mixtures A, B, and C contained 0.25, 1.0, and 2.0 mg., respectively, of glutathione in a total volume of 2 cc.

the effect is intermediate. These results are in harmony with the fact that with low glutathione concentrations the reaction rate remains constant until practically all the methylglyoxal has been converted into lactic acid, while with high glutathione the rate

tends to decrease slightly with time, particularly when an appreciable proportion of the methylglyoxal undergoes reaction. In the latter case, however, toxic effects undoubtedly enter the picture, so that too definite conclusions cannot be drawn.

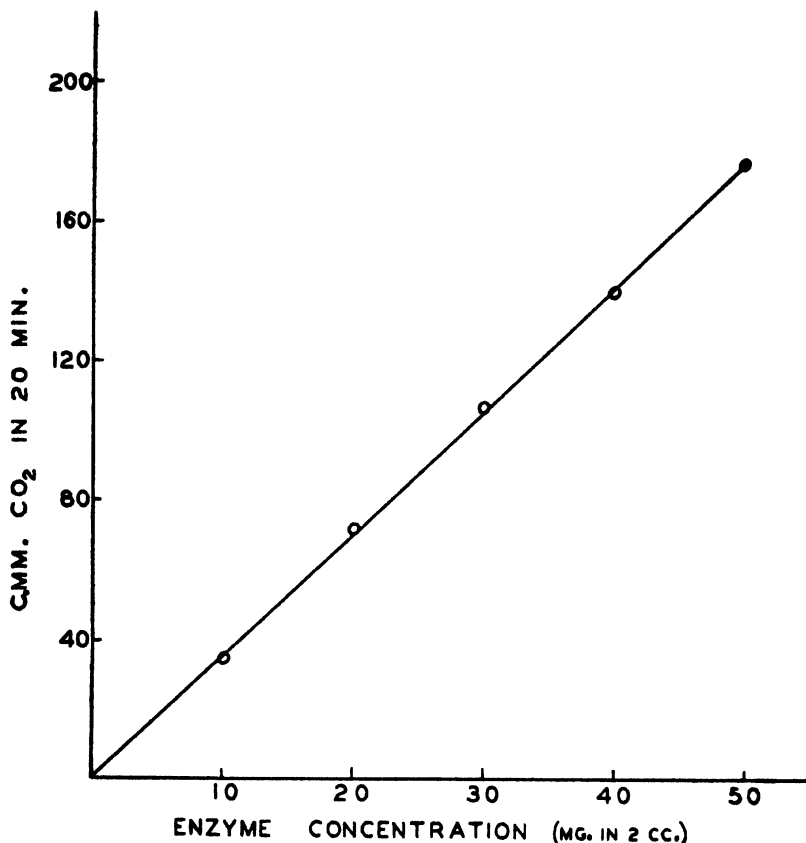


FIG. 4. Relation between enzyme concentration (amount of acetone-yeast) and activity.

*Relation between Activity and Enzyme Concentration*—Fig. 4 shows that direct proportionality exists between the quantity of enzyme used and the activity, as measured in terms of the volume of CO<sub>2</sub> evolved in 20 minutes. These results were obtained at 25° with washed yeast, in the presence of 0.25 mg. of glutathione, 2.0 mg.

of methylglyoxal, and 0.4 cc. of bicarbonate in a total volume of 2 cc. The glutathione was placed in the side bulb and the rest of the additions in the main chamber, and no corrections for yeast blank were made. The usual open period of 2 minutes was observed. In general, this straight line relationship was obtained only when the concentration of glutathione was held relatively low (below 0.5 mg.). With high glutathione concentrations irregular results were frequently obtained, the lower yeast concentrations being more active, and the higher less active than would be expected on the basis of a straight line relationship. Furthermore, with low glutathione concentrations, the activity is independent of the methylglyoxal concentration.

In methods for the quantitative determination of enzyme activity in cases involving an activator, it is generally the custom (16) to work with such an amount of activator that the maximum or full activity of the enzyme is obtained. For the reasons just mentioned, this is not practicable in this case; the amount of glutathione necessary to give maximum activity with 2 mg. of methylglyoxal is about 2.0 mg., and with this amount the straight line relationship between activity and concentration of enzyme no longer holds.

For the determination of the comparative glyoxalase activities of different acetone-yeast suspensions, the unit of glyoxalase may be defined as that amount which at 25°, in the presence of 0.25 mg. of glutathione and 2 mg. of methylglyoxal in a total volume of 2 cc., will produce in 20 minutes a total of, say, 100 c.mm. of CO<sub>2</sub>. Under these conditions, the amount of CO<sub>2</sub> obtained divided by 100 will give the number of enzyme units present. Experiments are now under way to determine the applicability of these conditions to the determination of tissue glyoxalase activity.

*Reaction between Glutathione and Methylglyoxal*—Jowett and Quastel (9) have reported several experiments which support the idea that glutathione reacts with methylglyoxal to form a complex compound which is then acted upon by glyoxalase with formation of lactic acid and regeneration of glutathione. Additional evidence for compound formation is supplied by the following experiment.

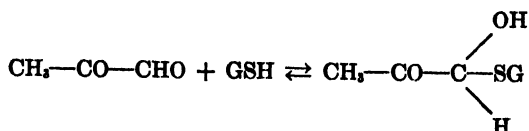
Solutions of pure glutathione and methylglyoxal were mixed in varying proportions, within the range of concentrations employed in the manometric work, and allowed to stand at room temperature.

At intervals portions were analyzed for sulfhydryl by titration with iodate in sulfosalicylic acid. Under these conditions methylglyoxal does not react with iodine. It was found that the amount of sulfhydryl present decreases very rapidly until it reaches a constant value, after which no further loss occurs. In the concentrations indicated in Table II this constant value was reached within 5 minutes after mixing, and no appreciable change occurred in 3 hours. It is probable, therefore, that under the conditions of our manometric experiments, this reaction will have been completed by the time readings are started. As is shown in Table II, the amount of sulfhydryl present at the completion of the reaction is dependent on the concentration of the reactants. Increasing the amount of methylglyoxal results in the reaction of a larger propor-

TABLE II  
*Reaction between Glutathione and Methylglyoxal*

GSH	Methylglyoxal	MeG per mole GSH	GSH at equilibrium	GSH reacting
<i>mg. per cc.</i>	<i>mg. per cc.</i>	<i>moles</i>	<i>per cent</i>	<i>per cent</i>
0.25	2.0	27	19.6	80.4
0.25	5.0	68	13.3	86.7
2.0	2.0	3.4	42.0	58.0
2.0	5.0	8.5	22.9	77.1

tion of the sulfhydryl. In other words, we are dealing with an equilibrium reaction, which on the basis of Quastel's suggestion, may be represented as follows:



The results of these experiments, while indicating the existence of an equilibrium, are not strictly applicable to those obtained in the manometric work. It is, therefore, our intention to defer the detailed discussion of the theoretical aspects of our work until we have carried out further experiments on the reaction between methylglyoxal and glutathione, and have completed a study of the

reaction kinetics of animal tissue glyoxalase. We wish, however, to point out several significant facts which, in our opinion, support the theory of complex formation.

As was shown in Fig. 3, with 0.25 mg. of glutathione the rate is independent of the methylglyoxal concentration. In this case, even the lowest concentration of methylglyoxal (1 mg.) represents an excess of 27:1 over the glutathione, assuming that they combine in molecular proportions. This may be sufficient to cause practically all of the glutathione to enter into combination, so that further increase in the methylglyoxal concentration will not greatly increase the amount of complex. Also with 2 mg. of glutathione, where the rate varies with the methylglyoxal concentration, the methylglyoxal is in excess, but the excess is only one-eighth as great as with the lower glutathione concentration. It seems reasonable to believe that under these conditions the proportion of glutathione in combination will depend much more on the concentration of methylglyoxal present. The results shown in Table II are in agreement with this idea. If from these figures the actual amounts of addition compound formed are calculated (assuming combination in molecular proportions), it will be seen that increasing the methylglyoxal concentration from 2 to 5 mg. in the presence of 0.25 mg. of glutathione results in increasing the actual amount of complex formed by only 0.02 mg. On the other hand, with 2 mg. of glutathione, the same increase in methylglyoxal concentration increases the actual amount of addition compound by 0.53 mg. Another interesting fact is that, with 2 mg. of glutathione, increasing the methylglyoxal concentration from 2 to 5 mg. results in increasing the activity by 35 per cent, while under similar conditions the amount of complex formed is increased by 33 per cent.

On the basis of these ideas, then, the true substrate would be the addition compound between glutathione and methylglyoxal, and the activity of the enzyme would depend upon, though not necessarily be directly proportional to, the concentration of the complex. It is difficult to understand why the rate of reaction should then be constant until all the methylglyoxal has been converted, as is shown to be the case in Fig. 1. Further experiments are in progress along this line.

*Iodoacetic Acid and Glyoxalase*—Dickens has recently published an article (6) in which he shows that the inhibiting effect of iodo-

acetic acid on rat liver glyoxalase is due entirely to its reaction with the activator, glutathione. When his preliminary communication on this subject (17) appeared, we had just completed some experiments with acetone-yeast glyoxalase which led to the same conclusion. In one series, unwashed acetone-yeast was allowed to stand at room temperature with excess iodoacetic acid (3.5 moles of iodoacetate per mole of glutathione as determined by titration) and at intervals the loss in glutathione and in glyoxalase activity was determined by the methods previously described. Although the glutathione method gives slightly high values, the loss in activity closely paralleled the loss in glutathione. Thus in 30 minutes the respective losses were 51 and 60 per cent; in 1 hour, 75 and 74 per cent; in 3 hours, 86 and 89 per cent. When the amount of glutathione originally present was restored, the glyoxalase was completely reactivated. In a second experiment, washed yeast was allowed to stand for 24 hours with the above amount of iodoacetate. After removal of the iodoacetate by washing, the yeast could be completely reactivated with glutathione, the enzyme itself not having been damaged.

#### SUMMARY

1. With acetone-yeast as the source of enzyme, the manometric method has been found to be suitable for the accurate determination of glyoxalase activity.

2. At 25° with low glutathione and methylglyoxal concentrations, the rate of the enzyme reaction is independent of the methylglyoxal concentration, and remains constant until all the methylglyoxal has been converted into lactic acid. Under these conditions, the rate is directly proportional to the amount of enzyme present.

3. With high glutathione concentrations, the rate is dependent on the methylglyoxal concentration, and is no longer directly proportional to the amount of enzyme.

4. In pure solution methylglyoxal reacts very rapidly with glutathione, the reaction apparently reaching an equilibrium. Evidence is presented in support of the idea that the complex thus formed is the true enzyme substrate.

5. Iodoacetic acid inhibits acetone-yeast glyoxalase by destroying the glutathione, the enzyme itself not being harmed.

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## THE SYNTHESIS OF RIBOSE-5-PHOSPHORIC ACID

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(Received for publication, December 14, 1933)

The existence of two isomeric forms of ribosenucleotides has been established very definitely in recent years and it has been claimed that the isomers differ not only in their chemical structure but also in their biological properties. The two isomeric forms differ from each other in the position of the phosphoryl group on the ribose component. In the case of muscle adenylic acid and of the muscle inosinic acid derived from it, the position of the phosphoryl group on carbon atom (5) of the ribose was formulated by Levene and Jacobs.<sup>1</sup> Robinson<sup>2</sup> claimed that the evidence advanced by these authors was in need of further substantiation and Levene and Mori<sup>3</sup> therefore furnished such evidence. For the final proof of the formulation of Levene and Jacobs,<sup>1</sup> it was desired to prepare ribose-5-phosphoric acid synthetically. This task has now been accomplished successfully and the synthetic ribose-5-phosphoric acid is found to be identical with the one obtained by Levene and Jacobs by hydrolysis of muscle inosinic acid.

For the synthesis of the substance advantage was taken of an observation made by us recently;<sup>4</sup> namely, that traces of methyl alcohol, present during the condensation of ribose with acetone, led to the formation of glycosidic derivatives of monoacetone ribose. Monoacetone methylribofuranoside was prepared by the

\* Commonwealth Fund Fellow.

<sup>1</sup> Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **41**, 2703 (1908); **44**, 746 (1911).

<sup>2</sup> Robinson, R., *Nature*, **120**, **44**, 656 (1927). Cf. Levene, P. A., *Nature*, **120**, 621 (1927).

<sup>3</sup> Levene, P. A., and Mori, T., *J. Biol. Chem.*, **81**, 215 (1929).

<sup>4</sup> Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **102**, 187 (1933).

condensation of ribose in a mixture of methyl alcohol and acetone in the presence of sulfuric acid. On methylation the monoacetone methylribofuranoside was converted into a monomethyl monoacetone methylribofuranoside. The monomethyl ribofuranose formed on hydrolysis of this compound was shown to be identical with 5-monomethyl ribofuranose by a comparison of the properties of their *p*-bromophenyl osazones. It therefore follows that the monoacetone methylriboside, which was employed to prepare ribosephosphoric acid, had the furanose ring structure and hence that the synthetic phosphate is ribose-5-phosphoric acid.

Phosphorylation of the monoacetone methylribofuranoside was carried out by means of phosphorus oxychloride in pyridine.<sup>5</sup> The final product was isolated in the form of the amorphous barium salt which had the correct composition and a specific rotation approaching that found by Levene and Jacobs.<sup>1</sup>

$$\begin{array}{l} \text{Natural,}^1 [\alpha]_D^{20} = +4.4^\circ \text{ (in water)} \\ \text{Synthetic, } [\alpha]_D^{20} = +5.99^\circ \text{ " " " " } \end{array}$$

By nucleating with a crystal of an old sample of the barium salt of the ribosephosphoric acid obtained from inosinic acid, the saturated aqueous solution of the synthetic barium ribosephosphate was induced to crystallize. This salt had the appearance of the crystalline salt previously prepared by Levene and Jacobs<sup>1</sup> and, like the latter, crystallized with 5 molecules of water of crystallization. Inasmuch as the specific rotation of the free acid from the crystalline salt had not been measured by Levene and Jacobs, a new sample of it was now prepared from inosinic acid and its rotation was compared with that of the synthetic acid. The specific rotations of the two were found practically identical.

$$\begin{array}{l} \text{Natural ribosephosphoric acid, } [\alpha]_D^{20} = +16.09^\circ \text{ (in water)} \\ \text{Synthetic " " " } [\alpha]_D^{20} = +16.54^\circ \text{ " " } \end{array}$$

Occasion was taken to follow the rate of hydrolysis of the phosphoryl group of 5-phosphoribose (Fig. 1). The hydrolysis was conducted in a solution of 0.01 N hydrochloric acid

<sup>5</sup> It may be mentioned in this place that the method of phosphorylation of 2, 3-monoacetone methylribofuranoside described in the present communication is being employed in this laboratory for the purpose of partial synthesis of inosinic acid and of other nucleotides of this type.

and the amount of free phosphoric acid formed after the elapse of various intervals of time estimated colorimetrically. The curve so obtained showed a very much smaller rate of hydrolysis of the phosphoryl group than in the case of 3-phosphoribose.<sup>6</sup> The two curves are comparable respectively to those obtained by Levene and Harris<sup>7</sup> for 3- and 5-phosphoribonic acids from natural sources.

Thus, final evidence is furnished as to the position of the phosphoryl group in inosinic and muscle adenylic acids.

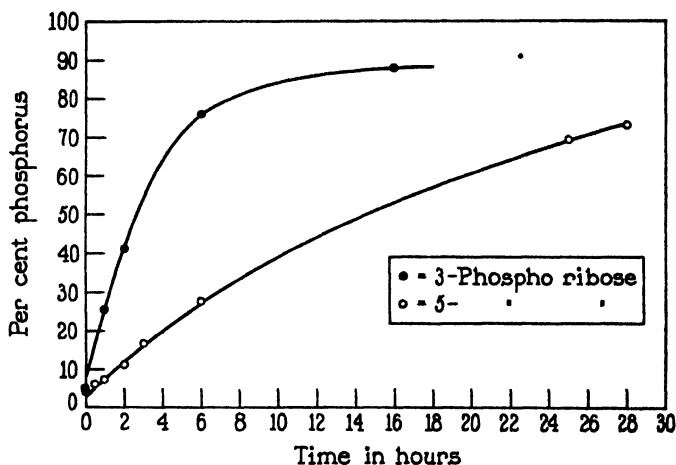


FIG. 1. Rates of phosphate hydrolysis of 3- and 5-phosphoribose

#### EXPERIMENTAL

*Preparation of Monoacetone Methylribofuranoside*—Dry, finely powdered *D*-ribose (20 gm.) was suspended in a mixture of acetone (380 cc.) and methyl alcohol (20 cc.) containing 0.2 per cent of sulfuric acid and the mixture shaken with anhydrous copper sulfate (40 gm.) for 20 hours at 37°. At the end of this period the solution did not reduce boiling Fehling's solution. The product was isolated as previously described for monoacetone ribose<sup>4</sup> giving a pale yellow, mobile syrup (yield 20.4 gm.). Fractional distillation gave, as the main fraction, 14 gm. of colorless mobile

<sup>6</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **101**, 419 (1933).

<sup>7</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **95**, 755 (1932).

syrup which distilled at 83–86° at 0.05 mm. It had the following composition.

5.220 mg. substance:	5.740 mg. AgI	
	$C_8H_{11}O_4 \cdot (OCH_3)$ .	Calculated. $OCH_3$ , 15.2
	(204.1)	Found. " 14.5

*Preparation of Monomethyl Monoacetone Methylribofuranoside*<sup>4</sup>—The monoacetone methylribofuranoside (10 gm.) was methylated by means of Purdie's reagents ( $Ag_2O$ , 34 gm.; MeI, 30 cc.) and the product, on isolation, was distilled under high vacuum. Yield, 9.2 gm. of a colorless, mobile syrup having a boiling point of 62–64° at 0.05 mm. and  $n_D^{25} = 1.4380$ .

*Preparation of Monomethylribofuranose*<sup>4</sup>—The methyl monoacetone methylribofuranoside (9.0 gm.) was dissolved in 0.04 N hydrochloric acid (120 cc.) and heated at 100° until the rotation became constant (in 125 minutes  $[\alpha]_D^{24} = +26.53^\circ$ ). The product was isolated as previously described.<sup>4</sup> Yield, 7.0 gm. of a pale yellow, viscous syrup.

*Preparation of p-Bromophenylosazone of Monomethyl Ribose*—The p-bromophenylosazone was prepared in the usual manner and was isolated in long, yellow needles.

Osazone	Melting point
Of Levene and Stiller <sup>8</sup> .....	177° (darkening)
New osazone . . . . .	175° "
Mixed.....	175–176° "

The substance had the following composition.

4.542 mg. substance:	0.454 cc. $N_2$ (20.5° at 762 mm.)
7.760 " " :	3.700 mg. AgI
	$C_{12}H_{13}O_2N_4Br_2$ . Calculated. N 11.2, $OCH_3$ , 6.2
	(499.02) Found. " 11.6, " 6.3

The specific rotation of the p-bromophenylosazone was

$$[\alpha]_D^{25} = \frac{-0.30^\circ \times 100}{0.5 \times 1.088} = -55.2^\circ \text{ (in absolute alcohol-pyridine, 3:2)}$$

*Phosphorylation of Monoacetone Methylribofuranoside*—A solution of 8.5 gm. of monoacetone methylribofuranoside in 34 cc. of

<sup>8</sup> The previously reported<sup>4</sup> melting point of 161–162° for this substance was raised to 177° by repeated crystallization from dilute methyl alcohol.

cold, dry pyridine (at  $-30^{\circ}$ ) was added in small portions, with vigorous stirring, to a solution of 4.1 cc. of phosphorus oxychloride in 52 cc. of cold dry pyridine (at  $-40^{\circ}$ ). The mixture was then transferred to an ice-salt bath for 2.5 hours, the temperature being kept below  $-10^{\circ}$ . The solution was now cooled to  $-40^{\circ}$  and 10 cc. of 90 per cent aqueous pyridine were slowly added with vigorous shaking, the temperature being maintained below  $-20^{\circ}$ . 20 cc. of water were then added and the solution adjusted to pH 8 by the addition of barium hydroxide solution. The small precipitate was removed by centrifuging in the presence of a little charcoal.

The solution was evaporated to a small volume under diminished pressure, at a temperature not exceeding  $45^{\circ}$ , in order to remove the pyridine. It was then diluted to 80 cc. with water and after the undissolved material (Fraction A) had been filtered off, 400 cc. of acetone were added. The precipitated barium chloride was filtered off and extracted four times with boiling 90 per cent acetone. On adding the extracts to the filtrate (Fraction B), a precipitate (Fraction C) formed. This was removed by filtration. The filtrate was then evaporated to dryness.

In order to hydrolyze the acetone and glycosidic residues, Fractions A, B, and C were separately dissolved in dilute sulfuric acid so that the final concentration of acid was 0.5 N and each solution was allowed to stand at room temperature for 20 hours. All three solutions were strongly reducing to boiling Fehling's solution at the end of this period. The products were separately isolated in the following manner.

The barium sulfate was filtered off, washed thoroughly, and the combined filtrate and washings neutralized with silver carbonate until no trace of halide remained in solution. The silver salts were removed by filtration, thoroughly washed with water, and the silver removed from the combined filtrate and washings by means of hydrogen sulfide. The excess hydrogen sulfide was removed by aeration and the silver sulfide filtered off. Barium carbonate was added until the solution was almost neutral and then barium hydroxide was added to pH 8.2. Carbon dioxide was immediately passed through the solution until neutral. The barium sulfate and barium carbonate were filtered off and the filtrate evaporated to small volume under diminished pressure. 4 to

5 volumes of alcohol were added to the filtrate and the white flocculent precipitate separated by centrifuging. It was washed by centrifuging with alcohol and ether successively and dried in a vacuum desiccator at room temperature.

The properties of the three samples of material are indicated in Table I.

Each of the three products was a white amorphous powder, dissolving slowly in water. They reduced Fehling's solution strongly, gave an orcinol test, and contained no inorganic phosphates.

TABLE I  
*Properties of the Three Fractions of Material*

Fraction	Weight	Found	
		Phosphorus	Barium
	<i>gm.</i>	<i>per cent</i>	<i>per cent</i>
A	2.1	7.73	37.75
B	4.0	6.12	32.00
C	2.5	8.38	34.24
C <sub>5</sub> H <sub>9</sub> O <sub>8</sub> PBa (365.45), calculated..		8.49	37.6

Fractions A and C were combined, dissolved in water, reprecipitated with alcohol, and dried as described above. The product was ground with a small quantity of water until it was almost all dissolved. The supernatant liquid was decanted; on seeding with an authentic specimen of barium 5-phosphoribose (from inosinic acid) and standing in the refrigerator at 0°, a crystalline deposit of colorless hexagonal plates formed. The crystalline material was freed from excess water by "drying" at room temperature in the presence of water vapor.

10.52 mg. substance lost 2.15 mg. at 80°

C<sub>5</sub>H<sub>9</sub>O<sub>8</sub>PBa · 5H<sub>2</sub>O. Calculated. Water of crystallization 19.78  
(455.45) Found. " " " 20.4

For analysis the substance was dried at 80°.

5.102 mg. substance: 29.280 mg. ammonium phosphomolybdate  
49.718 " " : 31.875 " BaSO<sub>4</sub>

C<sub>5</sub>H<sub>9</sub>O<sub>8</sub>PBa. Calculated. P 8.49, Ba 37.6  
(365.45) Found. " 8.33, " 37.72

The anhydrous material had the following rotation before crystallization.

$$[\alpha]_D^{25} = \frac{+0.22^\circ \times 100}{1 \times 3.674} = +5.99^\circ$$

0.1675 gm. of crystalline material was dissolved in 1.13 cc. of N hydrochloric acid and the solution diluted to 5 cc. with water.

$$[\alpha]_D^{25} = \frac{+0.44^\circ \times 100}{1 \times 2.660} = +16.54^\circ$$

*Hydrolysis of Barium Ribose-5-Phosphoric Acid*—25.45 mg. of hydrated salt (20.42 mg. of anhydrous salt = 1.73 mg. of phos-

TABLE II  
*Hydrolysis of 5-Phosphoribose*

Time	Free phosphorus
hrs.	per cent
0	4.0
0.5	6.0
1	7.2
2	11.1
3	16.8
6	27.3
25	69.5
28	73.2

phorus) were dissolved in 48.9 cc. of 0.01 N hydrochloric acid and 1.1 cc. of 0.1 N hydrochloric acid added in order to neutralize the barium. 5 cc. samples of this solution were sealed in tubes and heated at 100° in the water bath. After the elapse of various time intervals a tube was removed, rapidly cooled, and the contents centrifuged with a drop of 5 N sulfuric acid in order to remove the barium. 2.5 cc. of this solution were used in the colorimetric estimation. The results are recorded in Table II and in Fig. 1.

*Barium Ribose Phosphate from Inosinic Acid*—Amorphous barium ribose phosphate was prepared by the method of Levene and Jacobs,<sup>1</sup> from barium inosinate obtained from fish meat extract.

The material was crystallized from water, as previously de-



scribed. It was obtained in colorless, microscopic, hexagonal plates and had the following composition.

14.340 mg. substance lost 2.900 mg. at 80°

$C_5H_9O_8P\text{Ba} \cdot 5H_2O$ .	Calculated.	Water of crystallization	19.78
(455.45)	Found.	" " "	20.22

For analysis, the substance was dried at 80°.

5.840 mg. anhydrous salt: 33.290 mg. ammonium molybdate

46.972 " " " : 29.210 " barium sulfate

$C_5H_9O_8P\text{Ba}$ .	Calculated.	P	8.49,	Ba	37.6
(365.45)	Found.	"	8.22,	"	36.61

The free acid from the crystalline material had the following specific rotation, 0.1510 gm. of hydrated salt (0.1212 gm. of anhydrous salt) being dissolved in 1.0 cc. of N hydrochloric acid and diluted to 5 cc. with water.

$$[\alpha]_D^{25} = \frac{+0.39^\circ \times 100}{1 \times 2.424} = +16.09^\circ$$

## CHEMICAL STUDIES ON TOAD POISONS

### VI. CH'AN SU, THE DRIED VENOM OF THE CHINESE TOAD, AND THE SECRETION OF THE TROPICAL TOAD, BUFO MARINUS\*

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(Received for publication, November 13, 1933)

The isolation of cinobufagin from ch'an su (1), and of marinobufotoxin and bufagin from the secretion of *Bufo marinus* (2), has been previously described. As a result of further chemical study, the empirical formulæ originally proposed for these principles were revised to  $C_{25}H_{32}O_6$  for cinobufagin and  $C_{24}H_{32}O_6$  for bufagin (3). The average values of a number of analyses of these compounds are presented in the experimental portion of this paper. It will be noted that there is excellent agreement between the new formulæ and these data although the possibility of the further revision of the formulæ still exists.

In this paper we wish to present the results of the further chemical investigation of cinobufagin, bufagin, and marinobufotoxin.

Cinobufagin and bufagin are both lactones, forming hydroxy acids by the action of alcoholic sodium hydroxide. These acids have not been crystallized as yet although their analytical data indicate a considerable degree of purity. Coinciding with the hydrolysis of the lactone ring in cinobufagin, 1 mole of acetic acid (identified as the silver salt) is liberated, presumably from an acetoxy group. The cinobufaginic acid thus formed has been assigned the empirical formula  $C_{23}H_{32}O_6$ .

It has been previously reported that under the same conditions 1 mole of formic acid is liberated from bufagin (3). The evidence

\* Aided by a grant from the Committee on Scientific Research, American Medical Association.

now available, however, indicates that formaldehyde is the primary reaction product and that this then undergoes the familiar Cannizzaro's reaction in the alkaline medium. The origin of the

aldehyde, in all probability, is the grouping

$$\begin{array}{c} \text{—H}_2\text{C} \\ \quad \diagdown \\ \quad \text{C}=\text{CH}_2 \\ \quad \diagup \\ \text{—H}_2\text{C} \end{array}$$

is of interest to note that the presence of a similar group in ouabain or *g*-strophanthin has been recently demonstrated by Jacobs and Bigelow (4). The bufaginic acid which is formed during the alkaline hydrolysis of bufagin has the composition  $\text{C}_{22}\text{H}_{24}\text{O}_6$ . On prolonged alkaline treatment both cinobufagin and bufagin lose 1 mole of water. The resulting anhydro acids have not been isolated as yet in crystalline form.

The presence of two double bonds in cinobufagin and in bufagin is demonstrated by the formation of the corresponding tetrahydro derivatives on catalytic reduction. Simultaneously, small amounts of acidic substances are produced, probably by the opening of the lactone ring and subsequent formation of desoxy acids (5). Oxidation of cinobufagin with chromic acid gives a monoketone, indicating the presence of a secondary hydroxyl group. We have failed, however, to isolate any crystalline products from the similar oxidation of bufagin. On treatment with acetic anhydride both cinobufagin and bufagin form monoacetyl derivatives.

When bufagin is refluxed with 50 per cent alcohol containing 5 per cent of sulfuric acid, 2 moles of water are eliminated from the molecule. The dianhydrobufagin thus formed has the composition  $\text{C}_{24}\text{H}_{28}\text{O}_5$ . Under these comparatively mild conditions the methylene grouping in the molecule apparently is not affected, as there is no detectable formation of formaldehyde. However, on treating bufagin with 50 per cent sulfuric acid for 1 hour at  $70^\circ$ , formaldehyde is formed and may be identified after distillation from the reaction mixture. These facts are in complete accord

with the probable existence of the group

$$\begin{array}{c} \text{—H}_2\text{C} \\ \quad \diagdown \\ \quad \text{C}=\text{CH}_2 \\ \quad \diagup \\ \text{—H}_2\text{C} \end{array}$$

in the

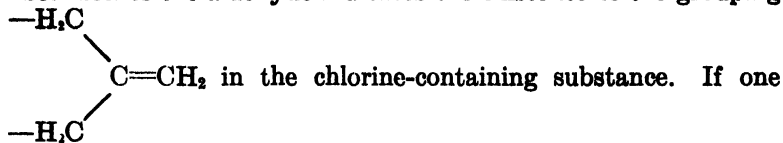
bufagin molecule. The yield of the dianhydrobufagin is rather poor. The reaction product is evidently a mixture of the mono- and dianhydro compounds, and the latter can be obtained in absolutely pure form only after numerous recrystallizations.

Cinobufagin, on treatment with hydrochloric acid, loses 1 molecule of acetic acid (which can be identified as the silver salt) and also, apparently, loses water. The hydroxyl groups involved in this dehydration are probably tertiary. However, no crystalline substances have been isolated from the reaction mixture as yet.

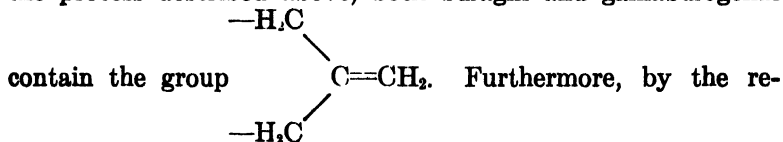
The analytical data for marinobufotoxin, which has been repeatedly recrystallized, correspond to the formula  $C_{38}H_{58}O_{10}N_4$ . It is difficult, however, to obtain the bufotoxins in an absolutely pure state. On acid hydrolysis of marinobufotoxin, suberic acid, arginine, and dianhydrobufagin are obtained; the latter compound being identical with that obtained from bufagin by acid treatment. Marinobufotoxin, therefore, may be grouped with the other bufotoxins; that is, compounds containing a typical bufagin coupled with suberylarginine.

In an earlier paper by Jensen and Chen (1) the isolation of a chlorine-containing substance from the mother liquor of cinobufagin was described. This compound was found to have the composition  $C_{24}H_{33}O_4Cl$ , and was regarded as a derivative of bufotalin. On the basis of the present experimental findings, however, it seems more probable that it is derived from gamabufogenin,  $C_{24}H_{34}O_6$ , which was first isolated from the secretion of the Japanese toad by Wieland and Vocke (6). Apparently, 1 mole of water has been removed from the gamabufogenin molecule with the simultaneous addition of a mole of hydrochloric acid. The compound is not affected by treatment with concentrated hydrochloric acid. Acetic anhydride gives an acetyl derivative of the composition  $C_{26}H_{34}O_6$ , 1 mole of hydrochloric acid being eliminated during the acetylation. On catalytic reduction of this acetyl derivative 3 moles of hydrogen are absorbed, as one would expect. The presence of a lactone group in the chlorine-containing substance is indicated by the formation of a hydroxy acid,  $C_{23}H_{33}O_4$ , on treatment of the compound with alcoholic sodium hydroxide. Simultaneously hydrochloric acid is split off and 1 mole of water is eliminated from the molecule. Formic acid can also be detected among the reaction products. The formation of formic acid here

is regarded as a secondary reaction as in the case of bufagin. The liberation of the aldehyde indicates the existence of the grouping



assumes that this compound is derived from gamabufogenin by the process described above, both bufagin and gamabufogenin



removal of the  $=\text{CH}_2$  group, both compounds would be converted into  $\text{C}_{23}$  derivatives. On acid hydrolysis gamabufogenin loses 1 mole of water (6) while bufagin loses 2; that is to say, gamabufogenin contains one tertiary hydroxyl group, and bufagin two. The difference of 1 mole of hydrogen between the composition of bufagin,  $\text{C}_{24}\text{H}_{32}\text{O}_6$ , and gamabufogenin,  $\text{C}_{24}\text{H}_{34}\text{O}_6$ , is thus explained.

The marked resemblance in the chemical behavior of the bufagins to that of the aglucones of certain plant glucosides has led to efforts to obtain definite proof of a chemical relationship between these two groups of compounds. At present an attempt is being made to demonstrate the existence of a 4-ring system in the bufagins similar to that of the sterols by the conversion of bufagin or cinobufagin into an acid of the composition  $\text{C}_{23}\text{H}_{38}\text{O}_2$ . It is hoped that the acid thus obtained will be identical with either norcholanic acid or norallocholanic acid.

Most of the analyses reported in this paper were carried out by Dr. Ing. A. Schoeller, Berlin-Schmargendorf, Germany. All compounds, if not otherwise specified, were dried for analysis at  $100^\circ$  in a high vacuum over  $\text{P}_2\text{O}_5$ .

#### EXPERIMENTAL

The revised empirical formulæ for bufagin, cinobufagin, their acetyl derivatives, and cinobufaginic acid are in excellent agreement with the following data. The preparation of all of these compounds has been previously described (1, 2).

			Carbon	Hy- drogen
Bufagin, m.p. 212–213°	$C_{24}H_{32}O_6$	Calculated	72.00	8.00
(Average of six determinations)		Found	71.89	8.05
Monoacetylbufagin, m.p. 203–204°	$C_{26}H_{34}O_6$	Calculated	70.59	7.69
(Average of four determinations)		Found	70.25	7.75
Cinobufagin, m.p. 222–223°	$C_{26}H_{32}O_6$	Calculated	70.10	7.50
(Average of seven determinations)		Found	70.02	7.66
Monoacetylcinobufagin, m.p. 195–196°	$C_{27}H_{34}O_7$	Calculated	68.94	7.24
(Average of five determinations)		Found	68.95	7.58
Cinobufagic acid, amorphous	$C_{24}H_{32}O_6$	Calculated	68.32	7.92
(Average of four determinations)		Found	68.25	8.19

*Tetrahydrobufagin*,  $C_{24}H_{36}O_6$ —1 gm. of bufagin was dissolved in alcohol and hydrogenated at atmospheric pressure in the presence of 0.3 gm. of palladium black. After 3 hours of shaking the reduction stopped, a little more than 2 moles of hydrogen being absorbed. The catalyst was filtered off and the alcoholic solution concentrated. A crystalline paste was obtained. The crystals were filtered off and recrystallized several times from dilute alcohol. The final product consisted of white prisms melting at 210–211°. The alcoholic filtrate from the crystalline paste was evaporated to dryness and stirred up with 0.1 N sodium carbonate. After filtering, the alkaline solution was acidified and extracted with ether. On evaporation the ether solution gave a product of acidic character which awaits further chemical investigation.

$C_{24}H_{36}O_6$ .	Calculated.	C 71.29, H 8.91
(Average of four determinations)	Found.	" 71.47, " 8.97

*Tetrahydrocinobufagin*,  $C_{25}H_{36}O_6$ —This compound was obtained by the catalytic hydrogenation of cinobufagin in a manner similar to that used in the preparation of tetrahydrobufagin. Tetrahydrocinobufagin was recrystallized several times from 80 per cent ethyl alcohol. The final product consisted of fine white prisms melting at 230°. Contrary to the experience of Kotake (7) we were able to obtain only one form of the reduced compound. We do not doubt, however, the possibility of the formation of stereoisomeric substances on reduction. The analytical data presented by Kotake are in agreement with the new proposed formula for this compound.

$C_{25}H_{36}O_6$ .	Calculated.	C 69.45, H 8.33
(Average of three determinations)	Found.	" 69.18, " 8.61

*Tetrahydrobufagin Monoacetate*,  $C_{26}H_{38}O_6$ —0.1 gm. of tetrahydrobufagin was heated with 2 cc. of acetic anhydride for 2 hours in a boiling water bath. After cooling, water was added and the solution let stand overnight. The acetyl derivative was filtered off and purified from dilute ethyl alcohol. The compound crystallized in fine white needles melting at  $213\text{--}214^\circ$ .

$C_{26}H_{38}O_6$ .	Calculated.	C 69.95, H 8.52
(Average of four determinations)	Found.	" 70.13, " 8.49

*Tetrahydrocinobufagin Monoacetate*,  $C_{27}H_{38}O_7$ —This compound was prepared from tetrahydrocinobufagin in a manner similar to that used in obtaining tetrahydrobufagin monoacetate. The product was purified from dilute ethyl alcohol, crystallizing in white prisms which melted at  $238^\circ$ .

$C_{27}H_{38}O_7$ .	Calculated.	C 68.35, H 8.02
(Average of three determinations)	Found.	" 68.53, " 8.37

*Bufaginic Acid*,  $C_{23}H_{34}O_6$ —0.4 gm. of bufagin was refluxed with 30 cc. of 1 N alcoholic sodium hydroxide for 3 hours. After cooling, the solution was diluted with water and let stand overnight. A small amount of flocculent material was then filtered off, and the filtrate made slightly acid with hydrochloric acid. After standing a day, the precipitate was filtered off and dried at room temperature. Formic acid could be identified in the filtrate. The bufaginic acid, however, could not be obtained in crystalline form. For further purification it was dissolved in ethyl alcohol (in which it is very soluble), and water added until a permanent turbidity resulted. The next day a pale yellow amorphous precipitate had settled out. This was filtered off and the filtrate further diluted with water. The precipitate thus obtained was filtered off, washed with water, and dried at room temperature. The product was an amorphous white powder. The acid began to decompose at  $180^\circ$ , and became progressively darker in color until it was completely melted at  $210^\circ$ . Water is probably split off at the higher temperatures. For analysis the compound was dried at room temperature *in vacuo* over calcium chloride.

$C_{23}H_{34}O_6$ .	Calculated.	C 67.98, H 8.37
(Average of three determinations)	Found.	" 68.00, " 8.33

*Detection of Acetyl Group in Cinobufagin*—0.4 gm. of cinobufagin was treated with alcoholic sodium hydroxide as in the preparation

of bufaginic acid. The precipitate obtained on acidification (hydrochloric acid) was filtered off and the filtrate distilled from an oil bath. The distillate (acidic towards litmus) was shaken with silver oxide and let stand overnight. The silver oxide was then filtered off and the filtrate evaporated *in vacuo* over sulfuric acid. The silver acetate was recrystallized from dilute alcohol.

$\text{CH}_3\text{COOAg}$ .	Calculated.	Ag 64.61
	Found.	" 64.45, 64.40

*Cinobufagone*,  $\text{C}_{25}\text{H}_{30}\text{O}_6$ —0.2 gm. of cinobufagin was dissolved in 4 cc. of glacial acetic acid and 0.3 cc. of a chromic acid solution (40 gm. of water, 8 gm. of sulfuric acid, and 5.3 gm. of chromium trioxide) was added with vigorous shaking. The solution was let stand for 10 minutes and then diluted with water. After standing overnight the precipitate which had formed was filtered off and purified by recrystallization from dilute ethyl alcohol. The product consisted of fine white needles melting at 240–241°.

$\text{C}_{25}\text{H}_{30}\text{O}_6$ .	Calculated.	C 70.42, H 7.04
(Average of three determinations)	Found.	" 70.30, " 7.32

*Dianhydrobufagin*,  $\text{C}_{24}\text{H}_{28}\text{O}_3$ —0.5 gm. of bufagin was refluxed for 2 hours on the water bath with 35 cc. of a 50 per cent alcoholic solution containing 5 gm. of sulfuric acid per 100 cc. of solution. The solution was let stand overnight in the ice box. The precipitate which had formed was then filtered off, dried, and purified from a very little ethyl alcohol. The product, however, was still not absolutely uniform, consisting mostly of fine needles which were slightly yellow. The crystalline material was repeatedly crystallized from alcohol and acetone. The final product consisted of fine needles possessing a yellowish tinge and melted at 245–246° with decomposition.

$\text{C}_{24}\text{H}_{28}\text{O}_3$ .	Calculated.	C 79.12, H 7.69
(Average of three determinations)	Found.	" 79.00, " 7.69

*Liberation of Formaldehyde from Bufagin on Acid Hydrolysis*—0.3 gm. of bufagin was heated with 5 cc. of 70 per cent sulfuric acid for 2 hours on a boiling water bath. The apparatus was connected with a condenser and a receiver containing ice water in order to prevent the loss of any formaldehyde volatilized during the hydrolysis. After cooling, the solution was carefully diluted with



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water and filtered. The filtrate was slowly distilled, the distillate being collected under ice water. The distillate gave a positive test for formaldehyde with resorcinol and sulfuric acid.

*Marinobufotoxin*,  $C_{32}H_{58}O_{10}N_4$ —The isolation of this principle from the secretion of *Bufo marinus* has been previously described (2). The material used for analysis was repeatedly crystallized from alcohol and from acetone. The final product consisted of clumps of fine white needles, melting at 204–205° with decomposition.

$C_{32}H_{58}O_{10}N_4$ .	Calculated.	C 62.46, H 7.95, N 7.67
(Average of two determinations)	Found.	" 62.65, " 8.21, " 7.61

*Hydrolysis of Marinobufotoxin*—0.5 gm. of marinobufotoxin was refluxed with 10 cc. of alcohol, 1 N with respect to hydrochloric acid, for 2 hours. After cooling, the solution was diluted with 10 cc. of water and let stand overnight in the ice box. The precipitate which had formed was then filtered off, recrystallized several times from dilute ethyl alcohol, and finally from acetone. This compound was found to be identical with the dianhydrobufagin obtained by the acid treatment of bufagin. The final product crystallized as needles, slightly tinged with yellow, melting at 240–241° with decomposition.

$C_{24}H_{38}O_8$ .	Calculated.	C 79.12, H 7.69
(Average of two determinations)	Found.	" 79.15, " 7.91

The filtrate from the dianhydrobufagin was evaporated to dryness *in vacuo*, the residue taken up in 2 N hydrochloric acid and heated for 5 hours in a boiling water bath. The solution was then decolorized with charcoal, filtered, and the filtrate evaporated to dryness. The residue thus obtained was repeatedly extracted with ether. On evaporation of the ether solution nearly pure suberic acid was obtained. The acid was recrystallized from water. The final product melted at 140° and showed no depression of the melting point when mixed with a sample of suberic acid.

$C_8H_{14}O_4$ .	Calculated.	C 55.17, H 8.04
(Average of two determinations)	Found.	" 55.30, " 8.03

The residue from the ether extraction was dissolved in 60 cc. of water, flavianic acid added, and the whole heated on the water

bath until a clear solution resulted. The solution was then placed in the ice box overnight. In the morning the arginine flavianate was filtered off and purified from water. The final product melted at  $265^{\circ}$  and showed no depression of the melting point when mixed with arginine flavianate.

$C_6H_{14}O_2N_4 \cdot C_{10}H_8(NO_2)_2OHSO_2H$ .	Calculated.	S 6.56
	Found.	" 6.36, 6.41

*Acetylanhydrogamabufagin*,  $C_{23}H_{34}O_5$ —1 gm. of gamabufagin chloride (1) was heated with 2 gm. of anhydrous sodium acetate and 15 cc. of acetic anhydride for 2 hours in a boiling water bath. After cooling, the solution was diluted with water and let stand overnight. The precipitate which had formed was filtered off. The presence of the chloride ion in the filtrate was observed. The precipitate was purified several times from dilute alcohol, crystallizing in leaflets of a slight yellow tinge, melting at  $225$ – $226^{\circ}$  with decomposition. The compound gave a negative test for chlorine.

$C_{26}H_{34}O_6$	Calculated.	C 73.24, H 7.98
(Average of seven determinations)	Found.	" 73.07, " 8.05

*Acetylhexahydroanhydrogamabufagin*,  $C_{23}H_{40}O_5$ —0.2 gm. of acetylanhydrogamabufagin was dissolved in alcohol and hydrogenated at atmospheric pressure in the presence of 0.2 gm. of palladium black. A little more than 3 moles of hydrogen were absorbed. After the catalyst was filtered off, the solution was treated in a manner similar to that used in preparing tetrahydrobufagin. Here, also, a reaction product of acidic character was obtained. The reduced compound was purified from dilute alcohol, crystallizing in white prisms melting at  $180^{\circ}$ .

$C_{26}H_{40}O_5$ .	Calculated.	C 72.22, H 9.25
(Average of two determinations)	Found.	" 72.34, " 9.06

*Anhydrogamabufaginic Acid*,  $C_{23}H_{32}O_4$ —1 gm. of gamabufagin chloride was refluxed with 10 cc. of a 25 per cent solution of potassium hydroxide in methyl alcohol for 3 hours. After cooling, the solution was diluted with water and let stand overnight. The precipitate which had formed was then filtered off. Formic acid and the chloride ion could be detected in the filtrate. After acidification (with acetic acid) the precipitate thus formed was collected

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and purified from acetone and from ethyl alcohol, crystallizing in white needles melting at 215° with decomposition.

$C_{21}H_{22}O_4$ .	Calculated.	C 74.20, H 8.60
(Average of two determinations)	Found.	" 73.77, " 8.95

All melting points are uncorrected.

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# CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS

## II. THE STEROLS OF MOLLUSKS

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From the unsaponifiable matter of the oyster, *Ostrea virginica*, I have isolated a sterol which closely resembles cholesterol; but its melting point could not be raised above 140° by repeated crystallization. While trying to purify the supposed cholesterol through the acetate, it was found that the acetate even after one recrystallization melted 15° higher than cholesteryl acetate, and that further recrystallization raised the melting point to 131–132°. The acetate is levorotatory,  $[\alpha]_D^{20} = -45.81^\circ$ . The sterol obtained from the acetate melts at 140°,  $[\alpha]_D^{20} = -43.90^\circ$ . It is evident therefore that this sterol is not cholesterol.

The steryl acetate adds bromine rapidly, giving a difficultly soluble bromide, m.p. 122°. The purified bromide was reduced with zinc dust to the acetate, which now melted at 134.5°,  $[\alpha]_D^{20} = -45.94^\circ$ , and the corresponding sterol at 142–143°,  $[\alpha]_D^{20} = -43.57^\circ$ . From the mother liquor of the bromide an acetate with a melting point of 133–134°,  $[\alpha]_D^{20} = -45.68^\circ$  and a sterol with a melting point of 142°,  $[\alpha]_D^{20} = -43.45^\circ$  were obtained. Comparison of the properties of the acetate and the sterol after purification over the bromide, with the starting material, shows that the latter was nearly pure.

From the purified sterol the benzoate and the propionate were prepared. The propionate melted at 113–114°, while the benzoate-like cholesteryl benzoate shows a double melting point. At 145–147° it melts to a turbid liquid which on further heating undergoes a play of colors, becoming clear at 152°. Since the properties of the sterol as well as its derivatives do not agree with those of any of the known sterols, it must be considered as a new

zoosterol, and I suggest the name *ostreasterol*, since it has first been isolated from the oyster.

The analysis of ostreasterol agrees with the formula  $C_{27}H_{46}O$  or  $C_{27}H_{44}O$ . The titration of the acetate with perbenzoic acid indicates the presence of two double bonds, which makes  $C_{27}H_{44}O$  the more probable formula. This is interesting because apart from lanosterol (1) and with the possible exception of stellasterol (2), zoosterols having more than one double bond have not yet been described.

The bromine addition product of ostreasteryl acetate, m.p.  $122^\circ$ , on analysis proved to be  $C_{27}H_{48}O_2Br_2$  or  $C_{27}H_{47}O_2Br_2$ . The entrance of 2 bromine atoms into the molecule can be reasonably explained through the addition of 1 molecule of bromine to a double bond. How the 3rd bromine atom has entered the molecule remains to be determined. Substitution may have taken place in some form, as occurs, for instance, during the formation of monobromospongosteryl acetate (3), monobromocholesteryl benzoate (4), and the lanosteryl bromides (1). Hydrogen bromide may have been added to another double linkage, or the bromide could be an insoluble addition product of 1 molecule of a dibromide and 1 molecule of a tetrabromide.

From the mother liquors of ostreasteryl acetate after long standing, a beautifully crystalline acetate separates, which after several recrystallizations melts at  $104-105^\circ$ ,  $[\alpha]_D^{21} = -17.23^\circ$ . Since it does not give an insoluble bromide, impurities consisting of ostreasteryl acetate could be removed by bromination. The purified acetate shows a melting point of  $104^\circ$  and an optical rotation of  $[\alpha]_D^{21} = -15.9^\circ$ . The sterol obtained from the acetate melts at  $122^\circ$ . The analysis of the acetate agrees with  $C_{27}H_{46}O \cdot CH_3CO$ . The sterol is entirely different from ostreasterol. Until the substance has been more thoroughly investigated, it will be referred to as Ostreasterol II.

Ostreasterol replaces cholesterol in all organs of the oyster. It has been isolated from the gills, the mantle, the muscle, and the so called liver. Since it was likely that other bivalves besides the oyster also contained ostreasterol, the unsaponifiable matter of the common round clam, *Venus mercenaria*, was examined. A sterol could be isolated from it, which after repeated recrystallization melted at  $139-140^\circ$ . Its pure acetate, m.p.  $131^\circ$ ,  $[\alpha]_D^{21} = -45.2^\circ$ , was identical with ostreasteryl acetate.

The discovery of ostreasterol in two different bivalves suggests that it is a sterol typical for the class of lamellibranchiates. Daniel and Doran (5), however, claim to have isolated cholesterol from the mussel, *Mytilus edulis*, but since the authors did not describe the properties of this sterol, it remains doubtful whether it actually was cholesterol, the more so, since I have shown that the mussel *Modiola*, which is very closely related to *Mytilus*, contains ostreasterol.

Whether ostreasterol is also present in fresh water bivalves remains to be seen. De Waele (6) claims to have isolated cholesterol from *Anodonta cygnea*.

In order to learn more about the distribution of ostreasterol among other classes of mollusks, the investigation was extended to members of the class of gastropods. Two investigators have shown that cholesterol is present in the fat of gastropods. Doree (7) found it in *Buccinum undatum*, the common whelk, and Leulier and Charnot (8) in a terrestrial gastropod, the common snail, *Helix pomatia*. In each case sufficient evidence has been given that the sterol is cholesterol.

On account of these reports one can assume that gastropods contain cholesterol. Since, however, it was not unlikely that they contained other sterols besides, for instance ostreasterol, I have studied the sterols of the large gastropods *Fulgur carica* and *Fulgur canaliculata*. The crude sterol melted between 139–143°, its acetate between 115–120°. Titration with perbenzoic acid showed 1.4 double bonds. The presence of a higher melting, more unsaturated substance in the acetate mixture was therefore indicated. By fractional recrystallization and purification through the bromide two sterols were isolated. One of them was cholesterol, identified through the properties of the sterol, its acetate, and its benzoate. The yield of cholesterol was rather low. The second sterol, the properties of which were very similar to those of ostreasterol, was present in a larger amount.

It must be mentioned here that the gastropods of the genus *Fulgur* feed largely upon oysters and other bivalves, from which food they may have acquired the ostreasterol present in their unsaponifiable matter.

The discovery of ostreasterol in the large class of bivalves and other mollusks shows that cholesterol is not universally distributed

throughout the animal kingdom. Our results indicate that in the study of the sterols of mollusks it is necessary to make frequent determinations of the melting points of the steryl acetates and titrate them with perbenzoic acid, in order to detect the presence of a high melting acetate of a sterol more unsaturated than cholesterol. The melting point and rotation of the sterol alone may lead to errors. Furthermore the distinction between fats and oils of plant and animal origin, which is based on the difference in the melting points of cholesteryl acetate and sitosteryl acetate, should be applied with caution.

Besides sterols the unsaponifiable matter of all mollusks investigated contains brown oils, which can be acetylated. The acetates can be distilled at 150–200° and 2 mm. The distillates show iodine numbers between 10 and 20. This excludes the presence of squalene-like substances. In the ice box the acetates crystallize but melt at room temperature. The products obtained by saponification of the acetate form colorless plates, which melt unsharply between 10 and 20°. The oil probably consists of a mixture of higher alcohols.

The author desires to express here his appreciation of the opportunity to cooperate with Professor Treat B. Johnson, Sterling Professor of Chemistry, in this new line of research which has been organized to increase our knowledge of the chemistry of marine products. He is also greatly indebted for the cordial cooperation of Mr. Howard W. Beach, President of the F. Mansfield and Sons, Oyster Dealers, New Haven, who has aided in the financial support of this work, and has supplied the oysters and other sea organisms used in this investigation.

#### EXPERIMENTAL

*Preparation of Unsaponifiable Matter*—6 kilos of oysters free from shells were passed through a meat grinder and extracted six times with 2 liters of acetone each time. The residue was then transferred to a large Soxhlet apparatus and extracted with ether for 24 hours. After that time a pure white residue remained. The acetone extracts were concentrated in a stream of carbon dioxide until the fat began to separate. The aqueous suspension remaining was extracted three times with ether and the ether

extracts were combined with those from the Soxhlet extraction. After drying over  $\text{Na}_2\text{SO}_4$  the extracts were evaporated to dryness in a stream of  $\text{CO}_2$ . The fat was then treated with 3 volumes of acetone to precipitate phosphatides and other acetone-insoluble material. After filtration the acetone was completely removed and the remaining fat dried in a vacuum desiccator for 48 hours. 91 gm. of a green semisolid fat were thus obtained, interspersed with plates of the appearance of cholesterol. The iodine number was 161.

For the saponification 94 gm. of fat were heated with 200 cc. of alcoholic potassium hydroxide (containing 200 gm. of KOH in 1 liter of 70 per cent alcohol) on the steam bath for 30 minutes. The solution was then mixed with 600 cc. of distilled water, transferred to a separatory funnel, and extracted first with 800 cc. of ether and then twice with 400 cc. After drying over anhydrous sodium sulfate the ether was removed by distillation. 10.1 gm. of a crystalline pinkish material remained, which was resubjected to a second saponification by heating it with 10 cc. of alcoholic potassium hydroxide for 15 minutes. The mixture was then diluted with 30 cc. of distilled water and extracted twice with 100 cc. of ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and distilled. A crystalline pinkish material was obtained, weighing 10.05 gm. or 10.7 per cent. The saponification, therefore, must have been complete.

*Isolation of Ostreasterol*—The sterol content of the unsaponifiable matter was 65.8 per cent, since 63.1 mg. gave 166.3 mg. of digitonide, corresponding to 41.5 mg. of sterol.

10 gm. of the unsaponifiable matter were refluxed with 20 cc. of acetic anhydride for 45 minutes. The mixture was allowed to cool; whereupon the acetates crystallized. After 3 hours they were filtered, washed with acetic anhydride and alcohol, dried in a vacuum desiccator, and weighed. The yield was 6.5 gm. The acetate was repeatedly recrystallized from alcohol. From time to time samples of the different fractions were withdrawn and saponified by refluxing them with 5 per cent alcoholic potassium hydroxide for 30 minutes. The resulting sterol was recrystallized twice from alcohol. The change of properties of the acetate and the sterol is recorded in Table I.

Preliminary investigations showed that the acetate adds bro-



mine rapidly, giving a rather insoluble addition product, which offered the possibility of further purification of the acetate (9). To a solution of 3 gm. of acetate in 15 cc. of dry ether 48 cc. of a 5 per cent solution of bromine in glacial acetic acid were added and the mixture was kept in the ice box for 3 hours. After that time 1.54 gm. of a fine colorless crystalline precipitate had separated, m.p. 120°. The mother liquor of the first precipitate was concentrated *in vacuo* until all the ether had been removed. Thereupon 1.5 gm. of a second crystalline precipitate was obtained, m.p. 118°.

TABLE I  
*Effects of Recrystallizations*

No of crystallisations	Acetate			Sterol		
	M p.	$[\alpha]_D$	Concentration	M p.	$[\alpha]_D$	Concentration
	°C.	degrees	per cent	°C.	degrees	per cent
3	129-130	-45.56	3.663	137-138	-42.83	3.63
7	130-131	-45.52	3.66			
10	131-132	-45.81	3.75	140	-43.90	2.05

TABLE II  
*Effects of Purification through the Bromide*

	Acetate			Sterol		
	M.p.	$[\alpha]_D$	Concentration	M.p.	$[\alpha]_D$	Concentration
	°C.	degrees	per cent	°C.	degrees	per cent
Fraction I. . . . .	134.5	-45.94	3.8	142-143	-43.57	1.8
“ II. . . . .	133-134.5	-45.68	0.74	142	-43.45	2.0
“ III. . . . .	127-130	-45.00	2.7			

The two precipitates as well as the remaining mother liquor were reduced separately by refluxing them with zinc dust in glacial acetic acid for 4 hours. After that time the solutions were filtered and distilled water was added to the warm solution until it turned slightly turbid. The acetate was then allowed to crystallize out, filtered, and recrystallized three times from alcohol. The properties of the acetates and the corresponding sterols obtained from the different fractions are given in Table II.

*Properties of Ostreasterol*—The purest ostreasterol showed a melt-

ing point of 142–143°. It gives the Liebermann-Burchard and the Salkowski reaction as cholesterol does. The solubility in different solvents is about the same as that of cholesterol. Under the microscope the crystals of ostreasterol obtained from 96 per cent alcohol appear in the form of flat needles. From methanol it crystallizes occasionally in the form of well shaped long needles.

*Rotation*—0.0537 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $-0.78^\circ$ ; hence  $[\alpha]_D^{20} = -43.57^\circ$ .

*Analysis*—Since ostreasterol contains water of crystallization which can be removed only with difficulty, it was dried at  $100^\circ$  and 1 mm. for 24 hours.

2.856 mg. gave 8.778 mg.  $\text{CO}_2$  and 3.03 mg.  $\text{H}_2\text{O}$

Found. C 83.85, H 11.90

Calculated for  $\text{C}_{27}\text{H}_{44}\text{O}$ . " 83.86, " 12.00

" "  $\text{C}_{27}\text{H}_{44}\text{O}$ . " 84.30, " 11.54

*Properties of Ostreasterol Acetate*—The acetate is easily soluble in ether and chloroform, less soluble in cold methyl and ethyl alcohol. Its solubility in warm methanol is greater than that of cholesteryl acetate. From dilute alcohol the ostreasterol acetate crystallizes in fine needles. The purest product showed a melting point of  $134.5^\circ$ .

*Rotation*—0.114 gm., 0.0478 gm. of substance dissolved in  $\text{CHCl}_3$  and made up to 3 cc. gave in a 1 dm. tube a reading of  $-1.746^\circ$ ,  $-0.73^\circ$ , hence  $[\alpha]_D^{20} = -45.94^\circ$ ,  $-45.97^\circ$ .

*Titration with Perbenzoic Acid*—Ostreasterol was titrated with perbenzoic acid in the usual manner. After 24 hours 0.2028 gm. of acetate had used 14.6 mg. of oxygen corresponding to 1.93 double bonds. After 48 hours, 0.2028 gm. of acetate had used 14.7 mg. of oxygen corresponding to 1.94 double bonds.

*Analysis*—2.785 mg. gave 8.301 mg.  $\text{CO}_2$  and 2.77 mg.  $\text{H}_2\text{O}$

Found. C 81.32, H 11.13

Calculated for  $\text{C}_{29}\text{H}_{48}\text{O}_2$ . " 81.24, " 11.29

" "  $\text{C}_{29}\text{H}_{48}\text{O}_2$ . " 81.62, " 10.87

*Addition of Bromine*—The first fraction of the bromide mentioned above was recrystallized three times from ether mixed with glacial acetic acid. The pure bromide forms small colorless blocks

or small needles, m.p. 122°, under decomposition. When exposed to light and air it decomposes.

*Analysis*—3.117 mg. gave 1.127 mg. Br

Found.	Br 36.16
Calculated for $C_{29}H_{41}O_2Br_2$ .	" 36.05
" " $C_{29}H_{47}O_2Br_2$ .	" 35.94

*Ostreasteryl Propionate*—Ostreasterol was refluxed with an excess of propionic anhydride for 45 minutes. The propionate, which separated after cooling was recrystallized five times from dilute alcohol. Small plates or needles, m.p. 113–114°, formed.

*Analysis*—4.561 mg. gave 13.65 mg.  $CO_2$  and 4.56 mg.  $H_2O$

Found.	C 81.76, H 11.19
Calculated for $C_{29}H_{48}O_2$ .	" 81.45, " 11.31
" " $C_{29}H_{46}O_2$ .	" 81.75, " 10.98

*Ostreasterol Benzoate*—To a solution of ostreasterol in dry pyridine an excess of benzoyl chloride was added and the mixture kept at room temperature for 3 hours. The benzoate was then precipitated with water, filtered, washed thoroughly with distilled water and alcohol, and then recrystallized five times from hot alcohol, in which it is more soluble than cholesteryl benzoate. The benzoate crystallizes in fine small needles. When heated in a capillary tube it melts at 145–147° to a turbid liquid, which on further heating turns blue at 150°, then emerald green, and becomes clear and colorless at 151–152°.

*Analysis*—4.719 mg. gave 14.41 mg.  $CO_2$  and 4.26 mg.  $H_2O$

Found.	C 83.30, H 10.10
Calculated for $C_{34}H_{48}O_2$ .	" 83.26, " 10.20
" " $C_{34}H_{46}O_2$ .	" 83.60, " 9.91

*Isolation of Ostreasterol II*—From the mother liquors of the ostreasterol acetate, which had not been purified through the bromide, after long standing beautiful needles, sometimes 2 to 3 cm. long, separate. They can be easily separated mechanically from impurities. After three recrystallizations this acetate melts at 104–105°.

*Rotation*—0.0357 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of 0.205°; hence  $[\alpha]_D^{21} = -17.23^\circ$ .

100 mg. of the acetate were dissolved in 1 cc. of absolute ether and to the solution 1.5 cc. of a 5 per cent solution of bromine in glacial acetic acid were added. After 18 hours standing in the ice box, only 3.7 mg. of a colorless, crystalline material, m.p. 110–120°, had separated. The mother liquor was then reduced with zinc dust in the usual manner. From the acetic acid the acetate crystallizes in beautiful clear prisms, m.p. 103–104°. After three recrystallizations from alcohol it showed a melting point of 104°.

*Rotation*—0.0262 gm. substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $-0.14^\circ$ ; hence  $[\alpha]_D^{21} = -15.9^\circ$ .

*Analysis*—4.641 mg. gave 4.73 mg.  $H_2O$  and 13.825 mg.  $CO_2$   
Found. C 81.30, H 11.41  
Calculated for  $C_{28}H_{44}O_2$ . " 81.24, " 11.29

The acetate was saponified in the usual manner and the sterol recrystallized three times from alcohol. During the three recrystallizations the melting point rose from 121° to 122°. *Ostreasterol* II crystallizes in plates. It gives the Liebermann-Burchard and Salkowski reactions, as does cholesterol.

*Isolation of Ostreasterol from Different Parts of the Oyster*—Oysters were divided into four different parts, the muscle, the mantle, the gills, and the remaining part of the body. The parts were then dried in a vacuum desiccator over concentrated  $H_2SO_4$  for 4 weeks. The dried material was ground to a powder and exhausted with ether in a Soxhlet apparatus. The ether extract was saponified in the usual manner, and the sterol part of the unsaponifiable matter precipitated with digitonin. The digitonide was then decomposed by refluxing it with acetic anhydride for 1 hour. The resulting acetate was recrystallized three times from alcohol. The results are recorded in Table III.

*Isolation of Ostreasterol from Venus mercenaria*—250 gm. of round clams free from shells were treated in the same manner as the oysters. 1.6811 gm. or 0.672 per cent of a green semisolid fat were obtained, which gave 0.2529 gm. of unsaponifiable matter or 14.9 per cent. The sterol part of the unsaponifiable matter was precipitated with digitonin, giving 0.6312 gm. of digitonide equal to 0.158 gm. of sterol or 62.45 per cent. The digitonide was refluxed with acetic anhydride in the usual manner and the resulting

steryl acetate recrystallized four times from alcohol. It melted at 130–131°. When mixed with ostreasteryl acetate of the same melting point, it melted at 130°. Through saponification ostreasterol melting at 139–140° was obtained.

*Isolation of Ostreasterol from Modiola*—From 161 gm. of *Modiola* free from shells, 1.6205 gm. of fat or 1 per cent were obtained. Saponification gave 0.2593 gm. of unsaponifiable matter or 16 per cent. It formed an orange crystalline material, which was acetylated, giving 0.1245 gm. of acetate. After five recrystallizations it melted at 131°. When mixed with ostreasterol acetate of the same melting point, it melted at 130–131°.

*Rotation*—0.0336 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $-0.556^\circ$ ; hence  $[\alpha]_D^{19} = -45.6^\circ$ .

TABLE III  
*Isolation of Ostreasterol from Different Parts of the Oyster*

	Amount of tissue	Fat		Unsaponifiable matter		M p of acetate
	gm	gm.	per cent	gm.	per cent	°C.
Muscle.....	9.277	0.1334	1.6	0.0842	63	129–130
Gills.....	5.5974	0.1551	2.77	0.0513	33.1	130
Mantle.....	4.8822	0.1221	2.50	0.0406	33.3	130
Rest.....	16.7665	1.6512	9.84	0.1945	11.8	128–129

From the acetate, ostreasterol, m.p. 140°, was obtained

*Sterols of Fulgur carica and Fulgur canaliculata*—From 4.5 kilos of the mollusks free from shells 75 gm. of fat or 1.7 per cent were obtained. 9.2 gm. or 12.25 per cent of the fat were unsaponifiable, and the unsaponifiable matter contained 32.8 per cent sterol. It was extracted with 70 cc. of 96 per cent alcohol each time and filtered. On the filter a dark brown oil remained. The filtrate was kept in the ice box for 12 hours, after which time 2.5 gm. of small yellowish prisms had separated (Fraction I), m.p. 139–143°. The material was acetylated, giving an acetate which after two recrystallizations from alcohol melted between 115–120°. 1 gm. of the acetate was dissolved in 10 cc. of dry ether and the solution mixed with 12.5 cc. of a solution of 5 per cent of bromine in glacial acetic acid. The mixture was kept in the ice box for 4 hours, after which time 0.2732 gm. of silky needles had separated, m.p. 113–

114°. The bromide was reduced with zinc in glacial acetic acid and the resulting acetate recrystallized twice from methanol. The acetate melts at 114.5–115°. A mixture of the acetate with cholesterol acetate melted at 114–115°. When saponified, it gave cholesterol, m.p. 148°. The cholesterol was benzoylated, the benzoate melting at 147° to a turbid liquid, which became clear at 175°.

The mother liquor of the bromide was then reduced with zinc in glacial acetic acid and the acetate recrystallized from alcohol. The melting point was 118–125°. Three recrystallizations raised it to 130–132°.

The mother liquor of Fraction I was concentrated and a second crop of sterol weighing 1.72 gm. collected, m.p. 125–130°.

*Rotation*—0.1088 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $-1.55^\circ$ ; hence  $[\alpha]_D^{19} = -42.73^\circ$ . The sterol was acetylated, giving an acetate which after one recrystallization showed the melting point 117–122°. It was titrated with perbenzoic acid. After 40 hours, 0.1677 gm. of substance had used 8.44 mg. of oxygen corresponding to 1.34 double bonds.

0.72 gm. of acetate were treated with bromine in the usual manner. After 2 hours in the ice box, 0.34 gm. of a brownish crystalline material, melting at 109–113°, had separated. The bromide was reduced and the acetate recrystallized three times from alcohol. The material melted unsharply at 116–118°.

The mother liquor of the bromide was reduced and the acetate recrystallized four times, m.p. 131–132°.

*Rotation*—0.1070 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $-1.60^\circ$ ; hence  $[\alpha]_D^{19} = -44.80^\circ$ .

When mixed with ostreasteryl acetate, m.p. 132°, it melted at 131°. Through saponification, a sterol of melting point 141° was obtained.

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## **SOME ANALYSES OF AZOPROTEINS: CASEIN, GELATIN, AND ZEIN COUPLED WITH ARSANILIC ACID**

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Early dyestuff patents<sup>1</sup> recognized the fact that diazonium compounds couple with proteins (15); but the subject was first systematically investigated by Pauly (31). Since the first classic paper by Landsteiner and Lampl (20) on the antigenic properties of such coupled proteins, which they called azoproteins, considerable work has been done with them. Klopstock and Selter (18) claimed that no combination took place, and that the solutions were simple mixtures; but Heidelberger and Kendall (14) showed that if alkali or buffer were present the diazonium compound actually coupled with the protein. Pauly found that two diazonium groups coupled with each histidine and tyrosine molecule, and none with the other amino acids tested. It has since usually been assumed that in coupling with proteins 2 molecules of diazonium compound similarly couple with each tyrosyl and each histidyl group present; but no attempt has been made to prove this. The use of arsanilic acid as the diazotizable amine seemed to offer a way of obtaining sufficient data to check this assumption.

Compounds of proteins with arsanilic acid were described in Landsteiner's paper, but no analytical figures were given. Reiner (33) coupled arsanilic acid with purified antipneumococcus sera, and found an  $\text{As}_2\text{O}_3:\text{N}$  ratio of 0.027 for one preparation, and 0.028 for another. Marrack (22) coupled arsanilic acid with pseudoglobulin and with crystalline ovalbumin. For two different preparations of the azoglobulin he found 1.1 and 2.54 per cent arsenic; for the azoalbumin he found 1.53 per cent. Haurowitz and Breinl's (13) product, obtained by coupling horse serum with

<sup>1</sup> German patent 82446, Klasse 8, July 22, 1894.



arsanilic acid, contained 4.87 per cent arsenic. Reiner (34) reported analyses on six preparations made from toxin and toxoid and arsanilic acid. His As:N ratios, expressed as ratios by weight, range from 0.253 to 0.940 for preparations made at different pH values.

In a number of studies on immunological specificity azoproteins have been, and promise to be, very helpful. It therefore becomes necessary to test Pauly's assumptions on which would be based estimates of the number of diazo groups that would be expected to enter a given protein. We have prepared and analyzed for nitrogen and arsenic several compounds of arsanilic acid with casein, gelatin, and zein.

#### EXPERIMENTAL

*Preparation of Compounds*—Arsanilic acid, prepared according to the directions of Lewis and Cheetham (21), was diazotized and coupled to the proteins in alkaline solution. The preparation of the casein and gelatin compounds, which was practically the same as that used by previous authors, has been described by the present writers elsewhere (17). Preparation of the zein compound differed chiefly in the omission of precipitation by alcohol.

*Casein-Arsanilic Acid*—In an effort to insure saturation, after analysis the protein compound was again treated with diazotized arsanilic acid (about 0.5 gm. per gm. of casein) as before, purified, and reanalyzed. This was repeated twice. The solutions still had the characteristic odor of casein, and yielded a specific precipitate with an anticasein serum.

After analysis the *gelatin-arsanilic acid* was treated again with diazotized arsanilic acid (about 0.5 gm. per gm. of gelatin), purified as before, and reanalyzed. These gelatin compounds were orange in the precipitated state, deep red in alkaline solution. The solutions gelled on cooling, sometimes becoming orange in color. Both solidification and color change were reversible by heat.

*Zein-Arsanilic Acid*—Zein was prepared from yellow maize meal by the methods of Osborne (Osborne (24), Chittenden and Osborne (4), Osborne, Leavenworth, and Brantlecht (29); see Abderhalden (2)). A 1 per cent solution in 0.2 per cent KOH was treated with a diazotized solution of arsanilic acid (about 2 gm. per gm. of zein) which had been neutralized by the addition of a sufficient

quantity of  $\text{Na}_2\text{PO}_4$  solution. These zein compounds were, like the others, orange in the precipitated state, deep red in alkaline solution. The precipitated compound was soluble in 80 per cent alcohol, giving an orange-red solution.

*Analytical Methods*—Nitrogen was determined by the usual Kjeldahl procedure. There might be some question whether all the diazo nitrogen could be estimated by this method, and Reiner (34) makes the assumption that none of the diazo nitrogen is recovered. In order to test this point, a diazo dye, 2,4-dihydroxy-azobenzene, and a dried preparation of casein-arsanilic acid were analyzed by the Kjeldahl method and also by the method of Dumas. The results indicated that though only part of the diazo nitrogen (about 50 per cent) is recovered from the dye by the ordinary Kjeldahl method, all of the nitrogen is thus recovered from the azoprotein. Arsenic was determined by the method of Ewins (7), which in the hands of Myers and DuMez (23) gave somewhat low results, but in the experience of the present authors yielded reproducible results of sufficient accuracy when tested with a variety of organic arsenic compounds of known purity. The same protein solution was always used for both determinations; and when possible the same pipette was used for measuring the samples.

### Calculations

The ratio by weight of arsenic to nitrogen which would be expected on the assumption that 2 molecules of diazonium compound couple with each tyrosyl and histidyl group in the protein molecule may be derived as follows: Let  $\theta$  stand for the expression  $(t/18,100 + h/15,500)$ , where  $t$  equals the per cent of tyrosine in the protein, etc. Then the number of gm. of arsenic combining with 1 gm. of protein is  $2 \times 75(t/18,100 + h/15,500)$  or  $150 \theta$ . This introduces  $28/75 \times 150 \theta$  or  $56 \theta$  gm. of nitrogen into each gm. of protein, which already contained  $1/F$  ( $F$  = the ratio of protein to nitrogen), making the total  $1/F + 56 \theta$  gm. of nitrogen. The ratio  $R = \text{As:N}$ , then, is

$$150 \theta F / (1 + 56 \theta F)$$

The reader will be able to verify likewise the following expressions.

$$\text{Per cent As (in compound)} = (150 \theta / (1 + 456 \theta)) \times 100$$

$$F_c \text{ (conversion factor for compound protein)} = (F + 456 \theta F) / (1 + 56 \theta F)$$

Independently of Pauly's assumption,

$$F_C \text{ obviously} = F(1 - 0.373 R) + 3.20 R$$

$N_M = 2 M\theta$  ( $N_M$  = number of entering groups per molecule;  $M$  = molecular weight of native protein)

$A = 434 \theta$  ( $A$  = gm. of arsanilic acid required to saturate 1 gm. of protein)

TABLE I

*Percentages of Tyrosine and Histidine According to Various Authors, and Values of  $\theta$  Calculated from Them*

Protein	Tyrosine	Histidine	$(\theta \times 10^4)^*$	Source of figures (bibliographic Nos.)
Casein	4.5	2.5	0.410	(1), (27)
	4.5	2.61	0.417	(10)
	5.36	2.6	0.464	(8)
	6.8	2.84	0.558	(9), (11)
Gelatin	Trace	0.4	0.026	(8), (12)
	0.25	0.53	0.048	(10)
	0.01	0.90	0.059	(5)
	0.25	1.0	0.079	(10), (32)
Zein	3.55	0.82	0.225	(26), (29)
	3.66	1.25	0.283	(10)
	5.2	0.8	0.339	(6), (19), (30)
	5.6	1.25	0.390	(8), (10)
Ovalbumin (crystalline)†	1.77	1.71	0.209	(28)
	2.35	2.3	0.278	(10)
	4.21	1.42	0.325	(3), (35)
	4.2	2.3	0.380	(8), (10)

\*  $\theta = (t/18,100) + (h/15,500)$ , where  $t$  = per cent tyrosine, etc.

† Figures for ovalbumin are included, though no work is here reported on this protein, for purposes of comparison, and for the convenience of those who may wish to use this immunologically most valuable protein.

The maximal expected value of the As:N ratio, when calculated from Pauly's assumption will obviously depend on the values used for  $F$  and  $\theta$ .  $F$  is not difficult to determine, and the values reported by different authors are fairly consistent, but the situation with  $\theta$ , which depends on the values for tyrosine and histidine content, is far otherwise. The best that can be done is to calculate several values for  $\theta$  from the various figures reported, omitting what seem obviously discrepant values, and thus define a range for  $\theta$  for each protein. The results for four common proteins are given in Table I.

For the factor  $F$  fairly concordant results can be found. We shall use these values, calculated from nitrogen determinations by the respective authors: casein, 6.40 (Osborne and Guest (27)), gelatin, 5.52 (Hitchcock (16)), zein, 6.19 (Osborne (4, 25)), ovalbumin, 6.62 (Calvery (3)). From  $\theta$  and  $F$  the range of the expected value of the ratio As:N can be calculated. This range, and our own analytical results, are given in Table II.

TABLE II

*Analytical Figures and Ratio by Weight of As:N in Compound Proteins*

Protein	Times treated with diazonium compound	Arsenic  <i>mg. per ml.</i>	Nitrogen  <i>mg. per ml.</i>	As: N	
				Found	Expected
Casein	1	0.453	1.18	0.383	0.343-0.447
	1	1.65	4.81	0.343	
		1.65	4.82		
	1	3.57	8.00	0.446	
		3.55			
	2	4.58	8.99	0.512	
		4.56			
	3	2.59	3.64	0.713	
		2.60	3.64		
Gelatin	1	0.411	12.63	0.032	0.021-0.064
			12.68		
	1	0.399	5.50	0.072	
		0.395			
	2	0.537	7.35	0.073	
Zein		0.537	7.35		0.193-0.338
	1	0.147	3.98	0.147	
	2	3.12	7.60	0.411	
		3.10			
	2	1.88	4.04	0.465	
	3	2.62	5.19	0.505	

Not only do the ratios, for the proteins treated repeatedly, surpass the highest of the expected values, but except possibly in the case of gelatin, they show no evidence of approach to a constancy indicative of saturation. The suspicion arises that some impurity of higher As:N ratio may have remained. Attempts to demonstrate this were inconclusive. Although each protein compound was purified before each analysis by several precipitations

in acid aqueous medium, and one or two in alcohol (except zein), two of them were subjected to further treatment. A casein-arsanilic acid compound was reprecipitated once in water and in alcohol twice. The ratio As:N changed from 0.512 to 0.506. A zein-arsanilic acid compound was subjected to three more precipitations in water. The ratio changed from 0.465 to 0.463.

It would seem that if the excess arsenic is due to an impurity, the impurity is firmly attached. Accordingly, experiments were carried out to see if arsanilic acid or a dye made from arsanilic acid (*p*-arsonic acid-azonaphthylamine) could be adsorbed on casein or casein-arsanilic acid. The proteins were treated in alkaline solution with about 0.5 gm. of arsanilic acid per gm. of protein, or of the dye about 0.15 gm. per gm. of protein, and allowed to

TABLE III

*Amounts of Arsenic Compounds Remaining on Proteins after Purification*

Protein	Arsenic compound	As N
Casein	0 (untreated)	0
	Arsanilic acid	0.014
	Dye	0.033
Casein-arsanilic acid	Before treatment	0.295
	Arsanilic acid	0.335
	Dye	0.293

stand overnight, then the protein was carried through the same series of precipitations as in the preparation of the azoproteins. The results are given in Table III. It will be seen that the amount of either arsenic compound remaining adsorbed on the proteins is quite insufficient to account for the discrepancies in question.

There remains the possibility that the diazoarsanilic acid may couple with some amino acid other than tyrosine or histidine. Pauly states that no coupling was observed with any of the amino acids tried by him, except these two, but does not give a list of the amino acids used. So the experiment summarized in Table IV was set up. To 0.25 mm of various amino acids, 0.50 mm of diazotized arsanilic acid and 1.7 mm of sodium carbonate were added, all at 5°. After 2 hours in the ice box the solutions were removed and compared in a colorimeter with a control solution

treated in exactly the same way, except that it contained no amino acid. The strengths of the various colors developed in terms of the control are given in Table IV. It will be seen that only one amino acid besides tyrosine and histidine gave even as much color as the control. Since this list is thought to include examples of every type of amino acid now known to be a usual constituent of proteins—the ones not tested all being very similar in structure to one or more of those used—it would seem that the experiment gives no support to the idea that coupling may occur with any other amino acids. The result with hydroxyproline, judged statistically, is significantly different from the results with the other

TABLE IV  
*Relative Amounts of Color Developed by Different Amino Acids with  
Diazotized Arsanilic Acid*

Amino acid	Relative color	Amino acid	Relative color
0 (control)	1 00	Histidine	2.00
Methionine	0 18	Tryptophane	0.33
Leucine	0 59	Phenylalanine	0.25
Alanine	0 25	Proline	0.37
Glycine	0.13	Hydroxyproline	1.00
Asparagine	0.59	Arginine	0.33
Serine	0.33	Cystine	0 23
Tyrosine	5.72	Glutamic acid	0 08

Mean (exclusive of tyrosine, histidine, and hydroxyproline) = 0.31 ( $\pm 0.11$ ).

amino acids, but since the color is no greater than that developed in the control, it is not likely that this indicates coupling with hydroxyproline. Besides, if coupling with some other amino acid were the explanation for the high values observed, we should expect the ratios, though higher than prediction, to reach and not to surpass some definite value. This is apparently not the case.

The authors are indebted to Mr. Harry Benson for assistance in this work.

#### SUMMARY

A series of compounds made by successive treatments of three proteins (casein, gelatin, zein) with diazotized arsanilic acid were

analyzed for arsenic and nitrogen, and the values obtained compared with those expected on the basis of Pauly's hypothesis that diazonium compounds couple with only the tyrosyl and histidyl groups of proteins. The results, while of the right general order of magnitude, are all too high, but it is not yet obvious whether this is evidence against the hypothesis. Formulas are given for calculating the number of azo groups introduced.

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# THE BASIC AMINO ACIDS OF KERATINS

## THE BASIC AMINO ACID CONTENT OF HUMAN FINGER NAILS AND CATTLE HORN

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(Received for publication, December 15, 1933)

A close chemical relationship of the proteins of many ectodermal tissues has been previously pointed out by Block and Vickery (1). They define a keratin as "a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalis, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine, and arginine that the molecular ratios of these amino acids are respectively approximately as 1:4:12." Analyses by Calvery (2) of egg-shell membrane and by Hess<sup>1</sup> of human finger nails are in agreement with this definition; on the other hand, the recent analysis of cattle horn by Abderhalden and Heyns (3) does not agree. Consequently this tissue has been reinvestigated; the results given below show that its composition closely resembles the keratins previously analyzed by Block and Vickery (1).

### EXPERIMENTAL

*Basic Amino Acids of Cattle Horn*—Shavings prepared from the outer layers of a single horn were digested with 200 volumes of 0.36 per cent hydrochloric acid containing a little pepsin (4). The residue was washed successively with boiling water, alcohol, and ether and dried at 110° for 4 weeks. The analysis of the basic amino acids was carried out by a slight modification of the method used previously (5). The protein was hydrolyzed with 8 N

\* Standard Brands Incorporated Fellow, 1932-34.

<sup>1</sup> Read before the meeting of the American Chemical Society at Chicago, September, 1933.

sulfuric acid and the resulting hydrolysate was adjusted to pH 5 to 6 with barium hydroxide. After removal of the barium sulfate, the histidine was precipitated directly from the amino acid mixture by silver nitrate and barium hydroxide at pH 7.4. The subsequent purification and isolation of the histidine were carried out in the customary manner (5).

The arginine was precipitated from the concentrated filtrate in the usual fashion (5).

Two modifications were made in the isolation of the lysine. The first was that the phosphotungstic acid was added at 90°, the hot

TABLE I  
*Amino Acids in Finger Nails and Cattle Horn*

Protein source	Investigator	Nitrogen	Histidine	Lysine	Arginine	Molecular ratios		
						Histidine	Lysine	Arginine
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>			
Finger nails..	Block	14.9	0.49	2.42	8.45 9.3*	1	5	15
“ “ ..	Hess		0.46	2.61	6.6	1	6	13
Cattle horn..	Block	15.1	0.64	2.76	9.42 9.8*	1	5	13
“ “ ..	Abderhalden and Heyns	18.6	2.3	4.0	2.0	1	1.85	0.78

\* Corrected for the solubility of arginine silver in baryta-silver oxide; i.e., 3.6 mg. of arginine per 100 cc.

solution was quickly cooled to 0° in an ice bath, and was allowed to remain at this temperature for 1 hour. The lysine phosphotungstate that had now separated was washed and then decomposed by amyl alcohol and ether (6). The resulting lysine sulfate solution was converted into the carbonate by first neutralizing almost all the excess acid with barium hydroxide and then by concentrating in the presence of a small amount of barium carbonate. The resulting solution is very satisfactory for the precipitation of lysine picrate<sup>2</sup> and no trouble was ever experienced with

<sup>2</sup> Commercial picric acid was purified by the method of Benedict (7) and recrystallized from benzene.

lysine dipicrate even if a significant excess of picric acid was added.

*Basic Amino Acids of Finger Nails*—Approximately 6 gm. of finger nail clippings were collected from one individual. These were successively extracted with hot water, alcohol, and ether. The dried material was then digested with pepsin-hydrochloric acid, reextracted with water, alcohol, and ether and finally dried to constant weight at 110°. The basic amino acids yielded by 4.36 gm. of the material were determined by the method of Vickery and Block (5). The results of this analysis are given in Table I.

#### SUMMARY

The determinations of the amino acids, arginine, histidine, and lysine, have been carried out on human finger nails and on cattle horn. The results indicate that these tissues have a chemical composition that resembles that of keratinoid tissues previously analyzed.

The method for the determination of the basic amino acids has been modified in several minor respects.

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## THE BASIC AMINO ACIDS OF SERUM PROTEINS

### II. THE EFFECT OF HEATING TO FIFTY-EIGHT DEGREES

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It has been shown (1) that the protein fractions obtained by precipitation of cattle serum with neutral salts vary in their amino acid composition. Determinations of histidine, arginine, and lysine were carried out on ten different protein fractions. These fractions were obtained by treating serum with various concentrations of ammonium sulfate, sodium sulfate, magnesium sulfate, and sodium chloride. The analyses indicated that these preparations all differed in amino acid composition. It appeared, however, that the more soluble serum proteins yielded the greater amounts of lysine. These results were tentatively explained on the hypothesis proposed by Sørensen (2) that the serum contains a number of components, possibly polypeptides, which are capable of association and dissociation to a varying degree. The chemical composition of the protein fractions precipitated by any physico-chemical treatment of serum is therefore due in part to the reagents employed.

The changes in the immunologic properties of a serum on heating have been empirically determined. Moll (3) showed in 1904 that if horse, dog, and rabbit sera were warmed for 1 hour at about 58° and then treated with an equal volume of saturated ammonium sulfate, there was an increase in the globulin precipitated and a corresponding decrease in the albumin.

Our experiments on cattle serum did not show any change in the amount of protein precipitated or in the basic amino acid composition of the albumin and globulin obtained from heated and unheated serum.

\* Standard Brands Incorporated Fellow, 1932-34.

## EXPERIMENTAL

*Preparation and Analysis of Serum Proteins*—The blood from one cow was allowed to stand in the ice box for 24 hours, the clot

TABLE I  
*Effect of Heating on Albumin-Globulin Ratio of Cattle Serum*

Serum	Albumin	Globulin
	gm.	gm.
Unheated . . . . .	12.0	27.0
Heated No. 1 . . . . .	9.2	26.3
“ “ 2 . . . . .	11.4	27.3

TABLE II  
*Basic Amino Acid Content of Heated and Unheated Serum Proteins*

Protein fraction	Nitrogen	Histidine	Arginine	Lysine*
	per cent	per cent	per cent	per cent
Unheated albumin . . . . .	14.4	1.94† 1.92‡	5.4† 4.2‡ 4.6§	9.5† 9.1† 10.2§
Heated “ . . . . .	14.4	2.01† 1.90‡	5.7† 4.8‡ 5.2§	9.6† 9.0‡ 9.9§
Unheated globulin . . . . .	14.2	0.88† 0.83‡	5.2† 4.4‡ 4.6§	6.2† 5.9‡ 6.9§
Heated “ . . . . .	14.2	0.78† 0.76‡	5.4† 4.8‡ 5.2§	6.0† 5.8‡ 7.1§

\* The highest value reported for lysine should be about 90 per cent of the lysine present in the original protein hydrolysate (unpublished experiments).

† Calculated from the nitrogen of the purified amino acid solution.

‡ Calculated from the weights of the crystalline salts, histidine dihydrochloride, arginine hydrochloride, and lysine picrate.

§ Corrected for the solubility of arginine silver or of lysine phosphotungstate. The solubility of lysine phosphotungstate in excess of the reagent has been found to be about 14 mg. of lysine per 100 cc. of solution (unpublished experiments).

was removed, and the remaining cells were centrifuged off. The serum was warmed to 22° and divided into three equal portions.

The first was treated with an equal volume of saturated ammonium sulfate. The second was warmed to 58° and maintained at that temperature for 1 hour, cooled quickly to 22°, and the protein was precipitated with ammonium sulfate in the same manner as the first sample. The third aliquot was warmed to 58°, maintained at this temperature for 3 hours, and was subsequently treated with an equal volume of saturated ammonium sulfate. The precipitation of the globulins was considered complete in 20 hours. The precipitates were filtered off, each was washed with 500 cc. of a one-half saturated ammonium sulfate solution, and the albumin and globulin were coagulated by heat after acidification to pH 4.5 and washed with hot water, alcohol, and ether. The protein fractions were dried at 110°. The yields of protein are summarized in Table I. The results indicate that under the conditions employed, there was no significant shift in the relative amount of albumin and globulin.

Several of these protein fractions were analyzed for their yield of the basic amino acids by the method recently described (4). In each analysis 4.6 gm. of protein were used. The results in Table II clearly indicate that the basic amino acid composition of the protein fractions is unchanged by heating the fresh serum.

#### SUMMARY AND CONCLUSION

If cattle serum is heated at 58° for 3 hours, the amount and the basic amino acid composition of the protein fraction precipitated by half saturation with ammonium sulfate are the same as are obtained from unheated serum.

The fractionation of cattle serum with half saturated ammonium sulfate supports our previous conclusion (1);<sup>1</sup> that is, the albumins or more soluble fractions yield the greater amounts of lysine on acid hydrolysis and the proteins obtained from blood serum are artificial products produced by the reagents employed in their preparation.

<sup>1</sup> Ettisch and Schulz (5) have prepared a serum albumin by half saturation with ammonium sulfate followed by dialysis and electrodialysis. They inferred from hydrogen electrode titration of this protein fraction that it contained 24.9 per cent of lysine. This value compares favorably with our previously reported figure of 26.9 per cent of lysine in the most soluble albumin (1).



I wish to thank Professor L. B. Mendel for suggesting this study.

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## THE BASIC AMINO ACIDS OF SERUM PROTEINS

### III. A CHEMICAL RELATIONSHIP BETWEEN SERUM PROTEINS OF VARIOUS ORIGINS

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The importance of a classification of proteins based on amino acid composition has been previously pointed out (1, 2). A measure of success has been achieved only with the keratinoid tissues and the protamines (3). The protamines, moreover, seem to form a special class of proteins. This leaves the keratins as the only true proteins which have been classified on a purely chemical basis. Block and Vickery (2) showed that keratins of widely differing origins yielded on acid hydrolysis amounts of histidine, lysine, and arginine in the molecular ratios of approximately 1:4:12 (2, 4). In every case the material analyzed comprised the entire protein portion of the tissue. In other words, the keratinoid tissues contained histidine, lysine, and arginine in a remarkably constant proportion although the actual percentage of these bases present in the tissue varied as much as 1000 per cent.

On the other hand, it has been indicated that the albumins and globulins obtained from cattle serum by precipitation with various neutral salts are not of constant basic amino acid composition but are simply artificial products produced by the reagents employed in their preparation. However, in every case, the more soluble protein fractions (albumins) obtained by this technique yielded more lysine than the less soluble fractions (globulins). These studies indicated that if the albumin-globulin determinations as carried out in the clinic were real estimations of the amounts of albumin and globulin present in a serum, then two sera with

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dissimilar albumin-globulin contents should yield unlike amounts of lysine. We have found that this is not the case.

Our previous investigations indicated that a better characterization of serum protein could be effected by a study of the whole protein rather than by attempting to isolate any "pure" preparation even though it might be crystalline.

#### EXPERIMENTAL

*Preparation of Serum Proteins*—The freshly drawn blood was centrifuged and a small aliquot of serum was removed for the estimation of total protein, albumin, and globulin by the phosphate method of Howe (5). The remainder of the serum was poured into 10 volumes of ice-cold acetone, allowed to stand at 4° for 5 hours, and the precipitate was centrifuged off. The protein was washed successively with hot acetone, benzene, alcohol, and ether. It was then dried at 110° for 24 hours. The ash content was not determined because of the small amounts of material at our disposal.

*Preparation of Urine Protein*—The urine was heated to boiling and dilute acetic acid was carefully added until no further precipitate occurred. The protein was washed with water, organic solvents, and dried at 110°.

*Determination of Basic Amino Acids*—Histidine, arginine, and lysine were isolated as the diflavanate, flavanate, and picrate respectively in the manner previously described (4).

#### Results

The analytical data are summarized in Table I. When these results are considered in the light of our previous investigations on cattle serum (1), we may draw the following conclusions.

1. Although the amount of total protein in various samples of blood serum may vary 100 per cent, its basic amino acid composition is constant.

2. In spite of the fact that our previous investigations have shown that the more soluble (albumin) fractions of the serum protein always contain the higher amounts of lysine, nevertheless even with 10-fold variations in the amount of albumin, the basic amino acid yield of the total serum protein remains the same.

3. The urinary protein from a patient with nephrosis closely

resembles the serum protein (6, 7) and is not "nearly all albumin" as has been stated in a recent text (8).

4. The basic amino acids yielded by acid hydrolysis of three kinds of mammalian serum proteins are constant both in absolute and relative amounts. Thus we have another instance of a tissue protein which, like the keratins, may be classified according to

TABLE I  
*Chemical Constitution of Serum Protein*

Source of protein	Amount of serum protein	Albumin	Globulin	Ratio albumin to globulin	Basic amino acids					Molecular ratio arginine to lysine
					Amount of protein hydrolysed	Nitrogen	Histidine	Arginine	Lysine	
	per cent	per cent	per cent		gm.	per cent	per cent	per cent	per cent	
Human serum, normal ♂	6.99	4.24	2.75	1.51:1	2.67	14.7	1.0	4.3	6.2	10:17
Human serum, normal ♀	7.09	4.41	2.68	1.58:1	2.67	14.7	1.0	4.5	6.9	10:18
Human serum, nephrosis, ♂	3.35	0.44	2.91	0.15:1	1.54	14.5	0.9	4.8	6.8	10:17
Human urine, nephrosis, ♂					3.00	15.2	0.9	4.4	6.9	10:18
Dog 1, serum, normal ♂	7.80	4.07	3.73	1.10:1	2.10	14.5	0.9	4.7	7.2	10:18
Dog 2, serum, normal ♂	6.88	4.29	2.59	1.65:1	2.14	14.7	0.7	4.9	7.3	10:18
Dog 3, serum, normal ♀	6.60	3.31	3.29	1.00:1	1.61	14.6	0.8	4.9	7.0	10:17
Cow serum, normal	7.80				9.20	14.2	1.2	4.8	6.9	10:17

the molecular ratios of the basic amino acids it yields on acid hydrolysis.

These observations should throw doubt on the idea that living serum contains two proteins or two groups of proteins which are usually classified as albumins and globulins. They support our previous suggestion (1) that the proteins obtained from blood serum by the usual physicochemical methods are not of a constant amino acid composition but are artificial products produced by

the reagents employed in their preparation (*cf.* (9)). Our studies on the serum proteins emphasize the idea of recognizing that products isolated from living tissue even by the mildest chemical procedures are not necessarily present as such in that tissue during life.

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## THE DISTRIBUTION OF A REDUCING SUBSTANCE (VITAMIN C) IN THE TISSUES OF FLUORINE- FED COWS\*

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The distribution of a reducing substance (vitamin C) in plant and animal tissues has recently assumed an important rôle in the study of vitamin C. Tillmans *et al.* (9) in an extensive series of researches made use of 2,6-dichlorophenolindophenol as the basis for the titration of a reducing substance whose characteristics seemed to be identical with those of vitamin C. The method used by Tillmans was modified later by Birch, Harris, and Ray (2) and subsequently by Bessey and King (1). The recent modifications of the Tillmans method have given us a rapid and direct method of titrating a reducing substance or substances in tissues, which has been shown by Bessey and King (1) to be in close agreement with biological assay for vitamin C.

In a previous report (4) it was suggested that chronic fluorine toxicosis is involved in some manner with the function of vitamin C, either by direct interference and inhibition, or by eliminating the mechanism through which vitamin C functions in the organism. This view rests upon the working hypothesis that chronic fluorine toxicosis causes a disturbance of cellular metabolism in such a manner as to interfere with normal oxidation and reduction processes. Further, certain data obtained in our studies on fluorine toxicosis in rats (5, 6) lend a limited amount of support to this hypothesis and suggest that further research is needed. Obviously, to submit this to a rigid test, experimental animals subject to scurvy are necessary. Such a study is now under way and it is hoped that more conclusive data can be obtained.

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Recently we had at our disposal Holstein dairy cattle which had been on a 5 year experiment involving the feeding of lime phosphate containing appreciable quantities of fluorine. Since no systematic study of the distribution of a reducing substance (vitamin C) has been reported for cattle, data were obtained upon certain organs and tissues with the object of ascertaining the normal distribution of vitamin C in the various tissues and the influence of chronic fluorine poisoning upon the vitamin C content of these tissues.

Because there are some indications that the chronic effect of fluorine poisoning is due to a disturbance in respiratory processes and because Szent-Györgyi (8) has shown that hexuronic acid (vitamin C) may function in certain oxidative mechanisms as a cyanide-inhibited oxygen carrier, data were obtained for the total cellular respiration as measured by oxygen uptake and the inhibitory effect of cyanide upon the tissue respiration.

#### EXPERIMENTAL

The animals available for study were Holstein cows which had been placed upon their experimental rations as 6 month-old calves in March, 1929, and were removed from the experiment in October and November, 1933. A total of eleven animals was available. Three of these received normal dairy rations adequate in energy and in all of the known dietary constituents, while the remaining eight cows were divided into three lots and in addition to the control rations received 0.022 per cent (Lot 4), 0.044 per cent (Lot 5), and 0.088 per cent (Lot 6) of the grain ration as fluorine fed as a mineral supplement in the form of raw lime phosphate. The cows were removed from the experiment individually to allow time for collecting whatever material was needed. The following organs and tissues were removed for vitamin C titration: anterior lobe of the hypophysis, posterior lobe of the hypophysis, suprarenal, suprarenal cortex, kidney (cortex), liver, heart muscle, striated muscle, smooth muscle from the uterus, smooth muscle (circular layer) from the duodenum, pancreas, thyroid, and mammary gland.

The tissues were removed as quickly as possible, ground with acid-extracted sand, extracted with four successive 10 cc. portions of 8 per cent trichloroacetic acid, and decanted through a

filter into a 50 cc. volumetric flask. The ground residue was next brought on the filter and washed with approximately 5 cc. of 8 per cent trichloroacetic acid. The contents of the flasks were then made up to volume and an aliquot titrated with freshly prepared 2,6-dichlorophenolindophenol following Bessey and King's procedure (1).

On tissues from a few of the organs the oxygen uptake and the inhibiting effect of cyanide on the oxygen uptake were measured. The method used for determining the oxygen uptake and the inhibiting effect of cyanide involves the use of the Barcroft differential respirometer and is fully described by Stare and Elvehjem (7). The measurements were conducted at 37° in a glucose-phosphate Ringer buffer solution at a pH of 7.3. The cyanide (NaCN) concentration used was  $M/300$ . Approximately 0.2 gm. of tissue was used for each determination. The tissues were taken directly from the abattoir to the laboratory and within 40 minutes from the time the animal was killed oxygen measurements were in progress. The organs first sampled for oxygen uptake were the liver, heart, kidney, and suprarenal gland. Early observations led us to believe that no significant changes would be encountered in the liver and heart and so experimentation was centered upon the kidney and suprarenal gland.

### *Results*

The results for the distribution of vitamin C are given in Table I. It is seen that the distribution of vitamin C in the various tissues and organs of the cow was widely variable. Minute quantities only were found in muscle. Striated muscle taken from the neck was uniformly low and represents the lowest value obtained for the tissues studied. Likewise, a low value was obtained with heart muscle. Smooth muscle on the other hand proved to be variable when taken from the myometrium. The values obtained for this muscle were 4 to 7 times higher than those obtained upon heart muscle. The unusually high values which were apparent for the uterine muscle caused us to remove sufficient smooth muscle from the circular muscle coat of the duodenum for vitamin C determination. The values obtained upon this muscle were found to be more nearly of the same order as those in the heart. It was noted that somewhat higher values seemed to



TABLE I  
Vitamin C in Various Organs and Tissues of Dairy Cows (Mg. per Gm. of Fresh Tissue)

Tissue	Basal ration				Lot 4, basal ration + 0.022 per cent F				Lot 5, basal ration + 0.044 per cent F				Lot 6, basal ration + 0.088 per cent F			
	Cow 57	Cow 60	Cow 62	Average	Cow 64	Cow 66	Cow 68	Average	Cow 67	Cow 68	Cow 69	Average	Cow 70	Cow 71	Average	
Striated muscle.....	0.017	0.020	0.016	0.018	0.010	0.036	0.013	0.020	0.020	0.01	0.010	0.013	0.013	0.016	0.015	
Heart .....	0.020	0.030	0.025	0.025	0.010	0.020	0.027	0.019	0.067	0.03	0.030	0.052	0.026	0.050	0.025	
Smooth " duodenum .....	0.020		0.065	0.043	0.040	0.040	0.050	0.043		0.03	0.030	0.030	0.070	0.10	0.085	
Uterine " .....	0.190	0.190	0.150	0.177	0.190	0.110	0.260	0.187	0.280	0.12	0.390	0.256	0.250	0.26	0.255	
Mammary gland.....	0.140		0.090	0.115	0.050	0.080	0.080	0.070			0.08		0.080	0.08	0.085	
Pancreas.....	0.140	0.120	0.120	0.126	0.120	0.130	0.130	0.126	0.150	0.13	0.150	0.143	0.050	0.12	0.085	
Kidney (cortex).....	0.10	0.130	0.080	0.103	0.080	0.090	0.120	0.096	0.120	0.14	0.150	0.136	0.140	0.17	0.155	
Thyroid.....	0.160		0.220	0.190	0.190	0.220	0.370	0.260	0.140	0.24	0.170	0.183	0.230	0.20	0.215	
Liver.....	0.140	0.180	0.430	0.250	0.190	0.20	0.30	0.230	0.210	0.31	0.230	0.250	0.370	0.37	0.370	
Hypophysis, posterior lobe .....	0.460		0.530	0.495	0.240	0.710	0.40	0.450			0.36		0.660	0.44	0.550	
Suprarenal.....	1.320		2.180	1.750	1.642	2.20	2.19	2.016	1.480	2.15		1.815	1.70	1.88	1.790	
" cortex .....	1.370		1.780	1.575	0.631	0.890	1.72	1.413			2.00		1.72	1.95	1.835	
Hypophysis, anterior lobe.....	1.950		1.600	1.805	0.881	2.50	1.17	1.10			1.93		1.78	2.65	2.215	
" .....									1.290	1.60						

prevail for the uterine muscle when large corpora lutea were present in the ovaries. This is interesting in view of the observation of Bessey and King to the effect that the corpus luteum was very high in vitamin C.

The thyroid gland, pancreas, mammary gland, liver, and kidney presented a group of organs whose vitamin C content was considerably higher than the range covered by muscle. The thyroid gland although variable in its range remained quite constant in its vitamin C content with respect to the influence of fluorine in the diet. The values for the pancreas are likewise rather constant. The mammary gland tends to be reduced in vitamin C content in the case of 0.088 per cent level of fluorine intake. That the low vitamin C content of secretory glands may influence the secretions was shown by the decrease of 25 to 30 per cent in the vitamin C content of the milk from the cows in Lot 6 (0.088 per cent fluorine) over the control animals. The liver and kidney on the other hand show a tendency toward increased amounts of vitamin C when 0.088 per cent fluorine was ingested. The same tendency was noted in the suprarenal cortex. The vitamin C content of the suprarenal cortex was very high, but we found the anterior lobe of the hypophysis was equally high if not higher, and tended to increase in the case of the cows on the higher fluorine intakes.

Table II gives the average results for the rate of respiration and the cyanide inhibition for the tissues studied. Liver tissue produced an oxygen uptake of between 200 and 300 c.mm. per gm. per hour with an average cyanide inhibition of 57 per cent. No significant difference was observed in the liver tissues of cows fed the various rations. The rate of oxygen uptake of kidney tissue averaged about 450 c.mm. per gm. per hour with a cyanide inhibition of 60 to 70 per cent. Here, likewise, no marked difference was observed from tissues taken from animals on the various diets. In our first determinations upon the suprarenal gland we used tissue containing both cortex and medulla and observed that the cyanide inhibition on the suprarenal gland from cows on the basal ration averaged 34 per cent, which is considerably lower than the usual 60 per cent cyanide inhibition (3, 7). The cyanide-inhibited respiration increased to 61 per cent on the suprarenal tissue from cows on the 0.044 per cent fluorine ration. This marked difference in the cyanide-inhibited fraction of the total

respiration led us to study separately the respiration of the medulla and cortex of the suprarenal gland.

The rate of oxygen uptake for the medulla varied from 209 to 244 c.mm. per gm. per hour and the cyanide inhibition from 41 to 53 per cent. The oxygen uptake of the cortical tissue was somewhat lower, ranging from 145 to 192 c.mm. per gm. per hour. The greatest difference was observed in the cyanide-inhibited respiration of the suprarenal cortex. This tissue from cows on the basal ration gave an average cyanide inhibition of only 16 per cent. The tissue of one cow from this group (Cow 62) showed

TABLE II

*Oxygen Uptake of Tissues of Dairy Cows (Average Values Reported in C. Mm. per Gm. of Fresh Tissue per Hour. Cyanide Inhibition Reported As Per Cent of Total)*

Tissue	Basal ration		Basal ration + 0.022 per cent F		Basal ration + 0.044 per cent F		Basal ration + 0.088 per cent F	
	O <sub>2</sub> up- take	CN inhibi- tion	O <sub>2</sub> up- take	CN inhibi- tion	O <sub>2</sub> up- take	CN inhibi- tion	O <sub>2</sub> up- take	CN inhibi- tion
Liver.....	212	58			281	55		
Heart.....	334	61						
Kidney.....	449	69	465	68	405	60	440	70
Suprarenal.....	216	34			194	61		
“ (medulla).....	209	48	244	41			226	53
“ (cortex).....	192	16	154	23			145	75

absolutely no cyanide inhibition. The cyanide inhibition of the cortical tissue from cows on the ration containing 0.022 per cent fluorine increased slightly to 23 per cent and on the high fluorine (0.088 per cent fluorine) ration increased to 75 per cent. The significance of this change in the cyanide-inhibited respiration of the cortical tissue of the suprarenal gland is not known.

It is seen from Table I that the vitamin C content of certain actively metabolizing tissues increases in those animals receiving excessive amounts of fluorine in their ration. This is observed in the kidney, liver, anterior lobe of the hypophysis, and the suprarenal cortex. From Table II it is seen that the cyanide-inhibited respiration of the suprarenal cortex also increased in the animals

receiving 0.088 per cent fluorine in the ration, but the total respiration decreased from an average of 192 to 145 c.mm. per gm. per hour, a decrease of 24 per cent. There appears to be a correlation between the presence of increased amounts of vitamin C and the presence of an increased cyanide-inhibited respiration factor or system in the suprarenal cortex of dairy cows suffering from fluorine toxicosis. This is to be anticipated in view of the conclusions reached by Szent-Györgyi (8) in regard to the function of hexuronic acid (vitamin C) in the animal body.

These observations, although confined by necessity to a small number of experimental animals, suggest that chronic fluorine toxicosis may be brought about by the inhibition of cellular respiration in certain essential actively metabolizing organs. It seems not unlikely that some phase of the aerobic respiration of cells is inhibited in the case of chronic fluorine poisoning, and it appears that a compensatory mechanism in which vitamin C is involved may be developed to maintain the oxidative processes of the tissues.

#### SUMMARY

The data presented show that vitamin C has a wide distribution in the animal body. It is particularly low in striated and heart muscle, and remarkably high in the suprarenal cortex and the anterior lobe of the hypophysis. The vitamin C content appears to be increased by fluorine toxicosis in certain actively metabolizing cells. An increase in vitamin C content was observed in the kidney, liver, anterior lobe of the hypophysis, and the suprarenal cortex when dairy cattle ingested 0.088 per cent fluorine with their grain mixture. Normal cellular respiration in the cortical tissue of the suprarenal gland of the cow is disturbed in chronic fluorine toxicosis. The total respiration is lowered as indicated by oxygen uptake measurements, and the anaerobic phase of respiration as indicated by the effect of cyanide upon the oxygen uptake is increased very markedly.

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# THE PREPARATION OF A CRYSTALLINE GLOBULIN FROM THE ALBUMIN FRACTION OF COW'S MILK\*

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It is generally recognized that the greater part of the coagulable protein of cow's milk may be separated as a fraction having the characteristic properties of an albumin. There seems, moreover, to be a wide-spread belief that this lactalbumin fraction may easily be obtained in a crystalline condition (1). Wickman (2), indeed, claimed to have obtained a crystalline protein by first preparing a solution of lactalbumin by the method described by Sebelien (3) and afterwards treating the solution according to the directions given by Gürber (4) for the preparation of crystalline serum albumin.

More recently, Sjogren and Svedberg (5) described in some detail a method by which a crystalline preparation of lactalbumin was obtained. We have made repeated attempts to follow the procedure of Svedberg but have never been successful. Svedberg's method is based upon that of Sørensen for the crystallization of serum and egg albumins and depends upon the separation of the protein from solutions containing high concentrations of salt. We have been unable to devise any method based upon the salting out of the protein which would yield a crystalline product from the lactalbumin fraction of milk. It may be noted in this connection that Chapman (6) records a similar failure after an extended study of the problem.

Although we have failed to crystallize lactalbumin by any modification of the methods which have been used successfully with other animal albumins, we have obtained from the albumin frac-

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tion, by a quite different procedure, a crystalline product with characteristics which differentiate it clearly from a true albumin. This product has been isolated repeatedly both from fresh skim milk and from a sample of commercial acid whey. The method now adopted consistently gives a uniformly crystalline product in amounts exceeding 50 per cent of the total albumin fraction and may be applied on a scale limited only by the facilities for handling large volumes of milk.

In the method described by Sjogren and Svedberg, skim milk is first half saturated with ammonium sulfate and the mixture brought to pH 5.2 by the addition of acetic acid. The precipitate of casein and globulin is removed by filtration and the salt content of the filtrate is increased to 80 per cent saturation. The resulting amorphous precipitate of albumin is redissolved in a convenient volume of water and submitted to crystallization at pH 5.2 after the method of Sørensen.<sup>1</sup> It occurred to us that our failure to confirm Svedberg's results might be due to the interference of traces of globulin or casein which had not been effectively removed in the first precipitation. Casein was, therefore, first precipitated by acidification with hydrochloric acid to pH 4.6. The pH of the whey was then readjusted to about 6.0 and the solution half saturated with ammonium sulfate to remove the major part of the globulin. The filtrate from the latter was next saturated with the salt and the precipitate of albumin redissolved in a small volume of water. This solution was adjusted to pH 5.2 and dialyzed in the cold room against frequent changes of distilled water. The sulfate reaction disappeared from the dialysate within a few days but no precipitate of protein appeared, nor was any formed when the solution was poured into an excess of distilled water. One may conclude, therefore, that no euglobulin was present. The dialysis was, however, continued for a longer period. After some 10 days a definite turbidity developed and a thick viscous oil accumulated. This oil was preserved. In a second experiment the dialysis was discontinued as soon as the turbidity was apparent. The solution was filtered and stored in the cold room. A crystalline precipitate began slowly to separate and

<sup>1</sup> In some instances we omitted the initial precipitation of the albumin fraction and attempted to crystallize the albumin directly out of the filtrate from the globulin-casein precipitate.

increased in amount over many days. These crystals grew to a remarkable size and would settle to the bottom of the flask within a few minutes after agitation of the contents (Fig. 1). When the oil which had been obtained in the first experiment was suspended in water and inoculated with the crystalline material, it crystallized completely within a few days. All solutions were saturated with toluene throughout the experiment

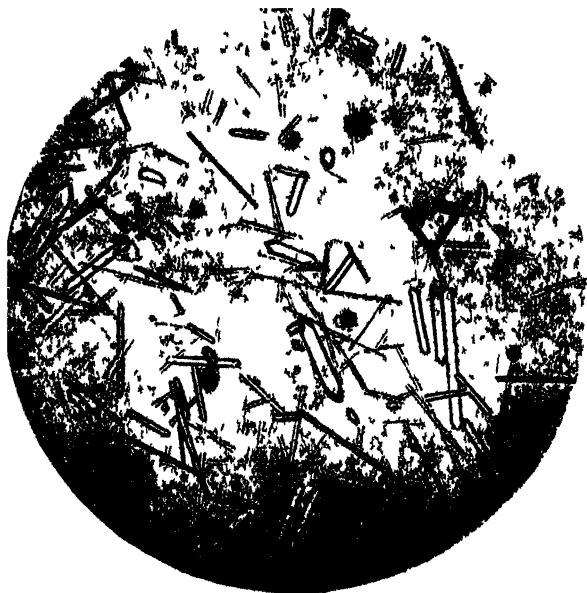


FIG. 1. Crystals obtained when the pH was adjusted to 5.2 previous to dialysis

*Routine Preparation*—In the preparation of the material in quantity certain modifications in the method have been found convenient

Milk whey contains only 4 to 5 gm. of coagulable protein per liter. It is obviously desirable, therefore, to employ some simple means of concentrating the whey before submitting it to procedures involving salting out and dialysis. We have found the simplest and safest method was one in which some two-thirds to three-quarters of the water was frozen out in an ice machine.



Sodium sulfate has been substituted for ammonium sulfate as a precipitating agent. This salt is employed exclusively in this laboratory in the routine preparation of crystalline egg and serum albumins. The advantages of its use in the fractional analysis of protein mixtures have been urged by Howe (7). To these may be added another of some importance when large quantities of the salt are being used. Owing to the low solubility of the salt at 0°, the greater part of that used may be recovered by allowing the mother liquors to crystallize in the cold room. The product, after recrystallization, may then be converted into the anhydrous salt by exposing in a thin layer to the air.

Two precautions may be noted regarding the use of sodium sulfate for the fractionation of protein mixtures. In the first place, it has been found that many samples of high grade sodium sulfate give distinctly alkaline solutions. When these are used in the salting out of protein the change of reaction which may occur should be controlled by the addition of dilute acid. The second precaution in the use of this salt depends upon an appreciation of the great increase in its solubility which accompanies an increase of temperature to 32°. At 20° a saturated solution is obtained when 21 gm. of anhydrous salt are dissolved in 100 cc. of water. Now the globulin fraction of milk whey is completely precipitated by the addition of 18 gm. of anhydrous salt per 100 cc. of whey. The removal of globulin may, accordingly, be effected at room temperature. For the complete removal of albumin from dilute solution, on the other hand, we add 36 gm. of salt to each 100 cc. of whey. To attain this solubility the solution must be maintained at a temperature approaching 30°. Both the precipitation and the filtration of the lactalbumin should, therefore, be conducted in a warm room. The description of a typical preparation follows.

The casein was removed from 30 liters of skim milk by the method of Van Slyke and Baker (8). After adjustment of the pH to 5.8, the whey was concentrated by freezing. The capacity of the ice machine which was in use is 16 liters. It was filled with whey and left overnight. In the morning the solid block, which was porous and friable, was broken up and spread on cloth stretched over the mouth of a large jar. This was placed in the cold room and the fluid which drained off was allowed to collect

until it amounted to about a quarter of the original volume of the whey. The residual snow was then melted and found to contain only traces of protein. A second charge was, meanwhile, introduced into the ice machine and concentrated in the same manner. The combined protein filtrates amounted to about 8 liters. The solution at this stage has, on occasion, been submitted to a second concentration by freezing, but this step is, perhaps, of little advantage.

The least soluble globulin was next precipitated by the addition of 18 gm. of anhydrous sodium sulfate per 100 cc. of concentrate. After a few hours the precipitate of globulin was removed by filtration and was discarded. The filtrate was warmed to 30° and a further amount of salt, equal to that used in the precipitation of the globulin, was added. When all the salt had dissolved, the moist precipitate, containing the whole of the residual protein, was removed by filtration in the warm room and rapidly scraped from the paper while still warm. This precipitate was then dissolved in about a liter of water to give a solution containing 8 to 10 per cent protein. When this solution was cooled to 0°, some sodium sulfate crystallized out. It was removed and washed. The solution and washings were combined and the pH readjusted to about 5.8 to 6.0. Toluene was added and the solution was dialyzed against frequent changes of distilled water in the cold room. At the end of 5 days the sulfate reaction in the dialysate was negligible. After an additional period of 7 days, dialysis was discontinued, the solution was filtered from traces of denatured protein, and the pH of the filtrate was adjusted to 5.2 by the controlled addition of 0.02 to 0.05 N hydrochloric acid, with methyl red as an external indicator. The solution became heavily clouded. It was seeded with the crystalline protein and left for several days at room temperature in the presence of toluene. The crystals obtained in this manner had the form pictured in Fig. 2. Seeding will hasten the separation but is not necessary, since the oil, which first separates, gradually assumes a crystalline form. Crystallization is more rapid at ordinary temperatures than at that of the cold room.

When, as in the preliminary experiments, the pH is adjusted to 5.2 prior to exhaustive dialysis, the crystals are of the form shown in Fig. 1. These seem the less stable type, since they will gradually change into those of Fig. 2 on long contact with water. This

change may be reversed if the latter crystals be dissolved in water, with the aid of a little salt, and dialyzed anew. Both forms of crystals will grow to unusual size. We have obtained them with a length of 3 mm.

Recrystallization may be effected by dissolving the crystalline material in water with the aid of sodium chloride, adjustment of the pH to 5.8 followed by dialysis, and then readjustment to pH



FIG. 2. Crystals obtained when the solution was dialyzed at pH 5.8 and crystallization induced by the addition of dilute acid.

5.2. If a maximum yield be sought, all mother liquors from the crystallizations may be treated by the addition of 35 gm. of anhydrous sodium sulfate per 100 cc., and the precipitate dissolved in a small volume of water and worked up after the manner of the main batch.

The crystalline material may be preserved in a moist condition, in the presence of toluene, in the cold room for many months. It still retains its ability to be recrystallized. The protein may be

dried by means of alcohol and ether to give a product which is still soluble in dilute solutions of salt, acid, or alkali. We have not attempted to recrystallize this dry material.

The yield of uniform crystalline material has been of the order of 1.5 gm. per liter of whey. This corresponds to 30 per cent of the total coagulable protein or to about 50 per cent of the lactalbumin fraction. When a careful attempt at quantitative isolation was made, this yield was increased to 1.8 gm. per liter of whey, or 60 per cent of the total lactalbumin fraction.

The problem of the nature of the residual protein must be left open for further investigation. The study of the solubility of the crystals, which is reported in a later section of this paper, would indicate that the material has a negligible solubility in the absence of salt. One might, therefore, expect crystallization after long dialysis to be complete and conclude that the residual protein must be different in character. Since the latter is soluble in water and in half saturated ammonium sulfate at pH 6.0, and is coagulated by heat, it may properly be called an albumin. When, however, one notes the enormous increase in solubility of the crystalline globulin-like material in the presence of quite small concentrations of ions, one may question whether crystallization is likely to result in a quantitative separation. The presence of only small amounts of soluble protein or other non-diffusible ions may well suffice to retain considerable amounts of the crystalline protein in solution.

An attempt to apply immunological tests to a solution of the problem has been inconclusive—perhaps because it has been somewhat premature. For the purpose of these tests, the mother liquor from an exhaustive crystallization was separated at pH 5.2, with sodium sulfate, into three fractions: Fraction A, the fraction precipitated by 25 gm. of salt per 100 cc. of protein solution; Fraction B, the fraction which was soluble at a salt concentration of 25 gm. per 100 cc, but precipitated by 28 gm per 100 cc.; Fraction C, the protein remaining after removal of Fractions A and B. To precipitate this last fraction, it was necessary to add a total of 35 gm. of salt per 100 cc of protein solution.

Fraction B was negligible in amount, indicating a break in the solubility curve of the mother liquor protein. Fractions A and C were twice precipitated and finally dialysed against distilled water until salt-free. A sample of twice crystallized globulin, together

with Fractions A and C, was used for the following immunological experiments. Guinea pigs were sensitized to one or other of these three preparations by the intraperitoneal injection of solutions containing, on the average, 40 mg. of protein. It was found that the sensitivity which subsequently developed in these animals was not specific for any one of the three fractions. That is to say, animals which were sensitized by means of Fraction A suffered anaphylactic shock when later injected intravenously with Fraction C or with the crystalline preparation. Cross reactions occurred between all three fractions. This procedure is, it will be agreed, a drastic test and cross reactions may be expected when only traces of the specific protein are present. Now we may, perhaps, hope to obtain the crystalline globulin as pure antigen by repeated crystallization, but the empirical basis of the fractionation of the mother liquor gives no confidence that even repeated salting out will suffice for immunological purification when such drastic tests are applied.

The fact that an apparent fractionation of the mother liquor can be made, at about 26 gm. of salt per 100 cc., suggests the presence of two protein components. The more soluble fraction, amounting to 25 per cent of the whole, has similar solubilities to the crystalline globulin and may be identical therewith. We were, however, unsuccessful in causing it to crystallize. The fraction separating between 21 and 25 gm. of salt per 100 cc. would then be the true albumin. Indeed, solubility measurements in concentrated salt solutions, in which the amount of mother liquor was varied, have indicated that the residual protein is most probably a mixture.

On the whole our impression is that the so called lactalbumin fraction consists of two distinct proteins. One has the characteristics of an albumin and has not been crystallized by us. The other, which has been obtained in a crystalline form and amounts to at least 60 per cent of the whole, has properties which would assign it to the group of globulins. This problem will be further investigated.

It is interesting to note that animals sensitized to cow serum did not react when injected with the crystalline globulin, although they gave reactions with the other fractions. On the other hand, animals sensitized to the crystalline globulin did give slight positive reactions with cow serum, indicating the presence of traces of

serum-like protein in the crystalline preparation. We feel that we may safely conclude that the crystalline globulin is not present in serum. This agrees with the general belief that the ordinary globulin fraction of milk and the corresponding fraction of serum contain a common antigen, but that the other fractions are distinct (9).

*General Properties*—The most distinctive property of this protein is its insolubility in water within the pH range 4.5 to 5.5. Within this range, however, it is freely soluble in the presence of surprisingly low concentrations of salt. At pH 5.2, for example, 0.2 per cent sodium chloride solution dissolves about 1 gm. per cent of the protein. It is soluble in saturated solutions of sodium chloride and in half saturated solutions of ammonium sulfate. It is completely precipitated by saturating its solution with ammonium sulfate. Thus the solubility of the protein in solutions of low salt concentration is similar to that of the globulins, whereas its solubility in high salt concentrations is comparable to that of animal albumins. Quantitative studies of solubility in low concentrations of sodium chloride and in high concentrations of sodium sulfate are reported in the next section.

The protein is completely precipitated from solution by such reagents as mercuric chloride or picric acid. It is insoluble in 50 per cent ethyl alcohol or acetone. At pH 5.2 it is rapidly and irreversibly coagulated at a temperature approaching 80°.

The biuret, xanthoproteic, glyoxylic acid, and Millon reactions are positive. The Molisch reaction, on the other hand, cannot be demonstrated, nor has any phosphorus been found. The ash content is negligible.

Total nitrogen, determined by the method of Kjeldahl (Arnold-Gunning modification), was 15.3 per cent. Colorimetric determination of cystine by the method of Folin and Marenzi (10) gave values approaching 4.0 per cent. A preliminary study of the nitrogen distribution gave values for the individual bases and for the non-amino nitrogen which were close to those reported for "lactalbumin" (11). The results of determination of the amide nitrogen and indirect (12) determination of the total dicarboxylic acids were close to those reported by Jones and Johns (13) for "lactalbumin." Until our analytical data are more extensive, we refrain from precise quotation of results.

It merits remark that these analytical data (11, 12) are referred to by their authors, and in standard text-books, as analyses of lactalbumin. They are, in fact, analyses of the total coagulable protein of cow's milk. Since the crystalline globulin, discussed in this paper, amounts to about one-half of the total coagulable protein, no wide differences should, perhaps, be expected between the nitrogen distributions of the two.

*Solubility*—As a test of the homogeneity of the product, the solubility in 0.1 per cent sodium chloride was determined in the presence of varying amounts of solid phase. Since a 4-fold increase in the amount of total solid failed to change the solubility, we conclude that no appreciable amount of foreign protein was present.

Three distinct preparations have shown a solubility of 66 to 68 mg. of protein nitrogen per 100 cc. of 0.1 per cent sodium chloride solution. When equilibrated with water at 30° these same preparations dissolved to the extent of 19.0 to 19.7 mg. of nitrogen per 100 cc.

In order to determine the quantitative effect of salt upon the solubility of the crystals, the latter were equilibrated at 32° for 24 hours with sodium chloride solutions of varying concentrations. The quantity of protein dissolved was then determined by the Kjeldahl nitrogen method. The solubility determinations in high concentrations of sodium sulfate were made in the same manner as in the experiments with sodium chloride, and the results of the two experiments are summarized in Table I. It will be seen that sodium sulfate is an effective precipitant when present to the extent of from 22 to 27 gm. per 100 cc. of water. In the case of the more dilute protein solutions, such as the unconcentrated whey, the effective range of salt concentration will be somewhat higher. A comparison of this precipitation range with the data of Howe (7), on the salt fractionation of the various milk proteins, indicates that the crystalline material clearly belongs to the fraction designated by Howe as the albumin fraction.

The above solubility data submit to rational analysis in terms of the ionic strengths of the salt solutions. In describing the solubility of oxyhemoglobin in dilute phosphate solutions, Cohn and Prentiss made use of the expanded form of the Debye-Hückel equation (14). Since, in the present case, it is possible to work

with more dilute solutions of electrolytes than did Cohn and Prentiss, we were content to apply the equation in its simplified form,  $\log S = 0.5 Z_1 Z_2 \sqrt{\mu} + \log S_0$ , where  $S$  is the solubility of the protein in salt solutions of ionic strength  $\mu$  (per 1000 gm. of water). The factor  $Z_1 Z_2$  may be taken to be a constant related to the apparent valence of the protein ions, and  $S_0$  is another constant representing the ideal solubility of the protein in the absence of salt.

TABLE I  
*Quantitative Effect of Salt upon Solubility of Crystals*

The mixtures were equilibrated at pH 5.2 and at 30° for 24 hours. The excess solid was centrifuged off and nitrogen determined by the Kjeldahl method on supernatant liquid. Gm. of nitrogen  $\times 6.53$  = gm. of protein.

Solution No.	Dilute sodium chloride solutions		Solution No.	High concentrations of sodium sulfate	
	Concentration of NaCl	Protein per 1000 gm. water		Anhydrous salt per 1000 gm. water	Protein per 1000 gm. water
	<i>M</i>	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>
1	0.00342	1.29	1	214.0	63.4
2	0.00684	1.60	2	225.0	18.8
3	0.0103	2.28	3	236.5	13.8
4	0.0137	2.91	4	248.0	9.17
5	0.0171	4.39	5	259.5	7.03
6	0.0197	5.12	6	271.0	5.02
7	0.0244	6.67			
8	0.0334	9.98			
9	0.050	23.7			
10	0.070	60.4			

When experimental values of  $\sqrt{\mu}$  are plotted against the logarithms of observed solubilities a straight line should result. The slope of this line defines the value of  $Z_1 Z_2$  and its intercept with the  $\log S$  ordinate gives  $\log S_0$ . This has been done in Fig. 3 for the salting in data with sodium chloride. The data are too limited to provide a valid test of the equation but so far as they go they indicate a limiting solubility of 0.3 gm. of protein per 1000 gm. of water. The slope of the line is approximately 9. In effect this indicates that the saturating substance (protein) behaves as an 18:1, 9:2, or a 6:3 valence type salt. The significance of this is uncertain.



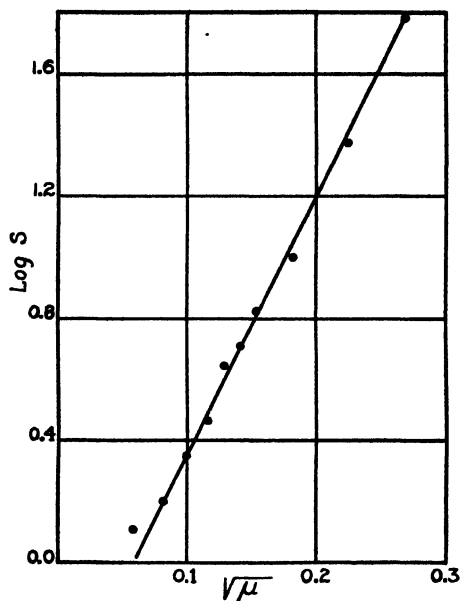


FIG. 3. Solubility of the protein in dilute sodium chloride solutions. Logarithm of the number of gm. of protein per 1000 gm. of water plotted against the square root of the ionic strength.

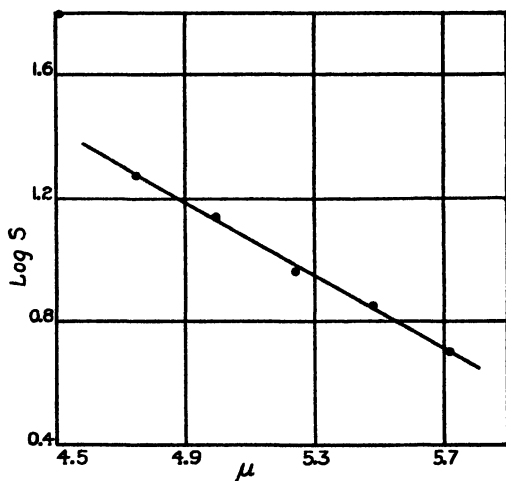


FIG. 4. Solubility of the protein in concentrated sodium sulfate solutions. Logarithm of the number of gm. of protein per 1000 gm. of water plotted against the ionic strength.

The extrapolated value for the solubility of the product in salt-free water does not agree with the experimental value previously reported in this paper. The fact that the experimental value is considerably larger than the calculated solubility may be due to the presence of very slight amounts of salt in the crystalline product.

In the analysis of the salting out data we may adopt the empirical salting out equation of Cohn (15),  $\log S = \beta - K_s\mu$ , where  $K_s$  is a salting out constant characteristic of the salt employed, and  $\beta$  is an intercept constant characteristic of the saturating substance.  $\mu$  again is the ionic strength per 1000 gm. of water. When  $\log S$  is plotted against  $\mu$  for the data relating to sodium sulfate in Table I, Fig. 4 is obtained.  $K_s$  has a value of 0.6 and the intercept constant  $\beta$  is 4.13.

The above results are offered at the present time merely as preliminary information. A more extensive investigation is in progress.

#### SUMMARY

A method is described whereby 60 per cent of the protein in the albumin fraction of cow's milk may be separated in a crystalline form by dialysis under controlled conditions. Since the protein is insoluble in salt-free water within the pH range 4.5 to 5.5, it should be classed as a globulin rather than an albumin. It is probable that a small amount of true albumin does exist in milk. This phase of the problem is now under investigation. A few quantitative observations of the solubility of the new protein in solutions of low and high salt concentrations are reported.

Acknowledgment is due Professor R. K. Cannan for his active interest and advice throughout this investigation.

To Dr. Bret Ratner we are indebted for the conduct of the immunological experiments reported herein.

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## TRYPTOPHANE METABOLISM

### IV. THE INFLUENCE OF OPTICAL ACTIVITY ON THE UTILIZATION OF TRYPTOPHANE FOR GROWTH AND FOR KYNURENIC ACID PRODUCTION\*

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In previous work from this laboratory on the relationship between optical isomerism and physiological response, *dl*-tryptophane was found quite as capable of supporting growth as the natural *l* component (Berg and Potgieter, 1931-32). As a normal outgrowth, these studies have been extended to include the resolution of *dl*-tryptophane (Berg, 1933) and the direct comparison of the *d* and *l* components from the standpoints of growth and kynurenic acid production. The observation of du Vigneaud and Sealock (1932) that acetyltryptophane is readily racemized unless certain precautions are observed in its preparation has necessitated repeating previous growth (Berg, Rose, and Marvel, 1929-30, *a*) and kynurenic acid studies (Berg, 1931) on this derivative. The present communication is, therefore, a comparison of free and acetylated *d*- and *l*-tryptophane as promoters of growth and of kynurenic acid production.

Since our first contribution was made, du Vigneaud, Sealock, and Van Etten (1932) have reported that free *d*-tryptophane can be utilized for growth, whereas its acetyl derivative cannot. The importance of the question justifies published confirmation and we are therefore presenting our data as briefly as possible, omitting certain aspects which should otherwise be considered in greater detail. The influence of the optical activity of tryptophane on its conversion into kynurenic acid has received no attention other

\* A portion of this communication was presented in abstract before the American Society of Biological Chemists at Philadelphia, April, 1932 (*J. Biol. Chem.*, **97**, lxxviii (1932)).

than that of Matsuoka, Takemura, and Yoshimatsu (1925) who found the output of this product considerably less after *dl*-tryptophane than after *l*-tryptophane administration. In view of the apparent ease in utilization of *dl*-tryptophane for growth, this observation seemed worthy of substantiation with experiments involving the direct use of the unnatural isomer, *d*-tryptophane.

#### EXPERIMENTAL

The free and acetylated *l*-tryptophane, *dl*-tryptophane, and *d*-tryptophane were all prepared in this laboratory according to methods previously summarized (Berg and Potgieter, 1931-32; Berg, 1933). Their purity was checked by melting point determinations and by elementary (nitrogen) and optical analyses.

*Growth Studies*—In testing the compounds for growth-promoting ability they were incorporated in a tryptophane-deficient basal ration (composed of acid-hydrolyzed casein (see Berg and Rose, 1929) 14.7, cystine 0.3, starch 40, sucrose 15, Crisco 19, cod liver oil 5, salt mixture 4 (Osborne and Mendel, 1919), and agar 2 per cent), in which they replaced an equal percentage of hydrolyzed casein. This food mixture was fed *ad libitum* to albino rats housed in individual false bottom cages. Twice daily each animal received as supplement a pill containing 100 mg. of yeast<sup>1</sup> and 50 mg. of starch. Water was always available.

A litter of nine rats was used in studying the efficacy of *d*-tryptophane as a growth promoter. One of these served as a control, receiving the unsupplemented tryptophane-deficient diet throughout. The remaining eight were separated into four pairs. During the first 40 days each pair received 0.2 per cent and 0.1 per cent of *d*- or of *l*-tryptophane respectively. During the second 40 days the *d*-tryptophane and *l*-tryptophane feedings were reversed. The growth rates are summarized in Chart I. Food consumption records are omitted in order to conserve space. An attempt has been made to correlate growth and food consumption data by employing, as previously (Berg and Potgieter, 1931-32), the efficiency quotient of Palmer and Kennedy (1931). This quotient, as we have modified it for our use, represents the food consumed in gm. per gm. of gain in weight per 100 gm. of body

<sup>1</sup>The yeast was kindly supplied by the Northwestern Yeast Company.

weight. The smaller quotient thus represents the more efficient utilization of food for growth. The strain of animals used in these

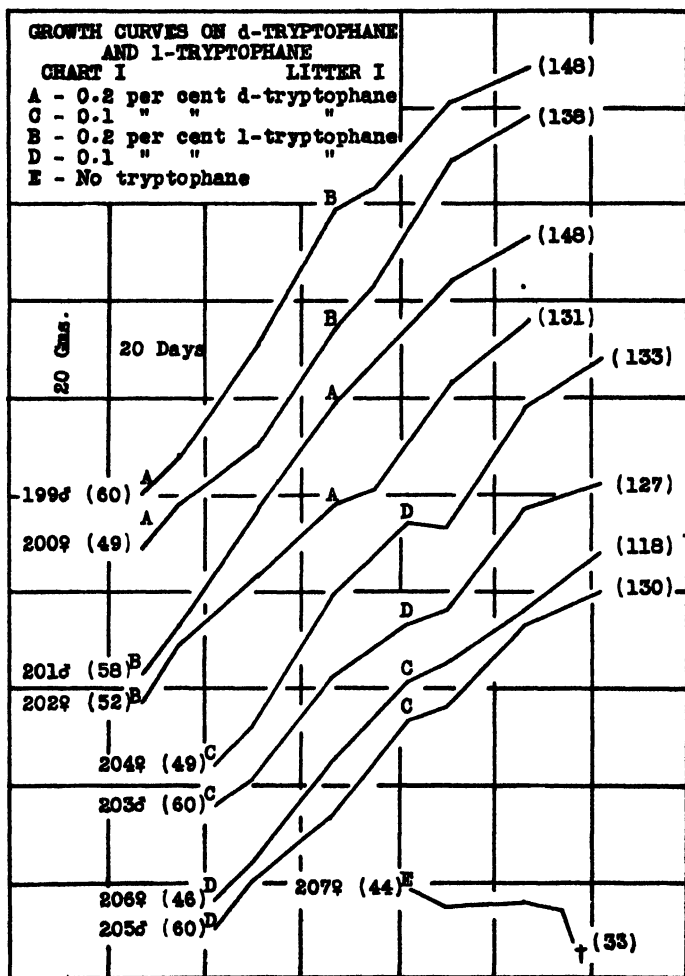


CHART I. The initial and final weights of the rats are given in parentheses. The dagger indicates the death of the animal.

studies was obviously slower growing and hence the individuals show generally higher efficiency quotients than those previously

obtained. Individual and average efficiency quotients are presented in Table I. Although there is a fairly wide variation in individual values, the averages are in close agreement and substantiate the growth curve indications. The rat can utilize either free isomer of tryptophane equally well for growth.

The technique employed in the acetyltryptophane studies was essentially the same as that above, except that no changes were made in the diets during the 80 day period. Two litters of animals were used. Of the first, one control received 0.15 per cent and two,

TABLE I  
*Comparison of Efficiency Quotients*

Rat No and sex	<i>l</i> -Tryptophane		<i>l</i> Tryptophane efficiency quotient	<i>d</i> -Tryptophane		<i>d</i> -Tryptophane efficiency quotient
	<i>per cent</i>	<i>days</i>		<i>per cent</i>	<i>days</i>	
199 ♂	0.2	41-80	5.16	0.2	1-40	4.05
201 ♂	0.2	1-40	4.32	0.2	41-80	4.62
200 ♀	0.2	41-80	4.11	0.2	1-40	6.35
202 ♀	0.2	1-40	6.03	0.2	41-80	5.01
Average of 40 day periods			4.91			5.01
203 ♂	0.1	41-80	5.40	0.1	1-40	6.48
205 ♂	0.1	1-40	5.71	0.1	41-80	5.32
204 ♀	0.1	41-80	4.93	0.1	1-40	4.82
206 ♀	0.1	1-40	6.32	0.1	41-80	6.45
Average of 40 day periods			5.59			5.77
" " averages			5.25			5.39

0.2 per cent of *l*-tryptophane; three pairs received 0.246 per cent of the acetyl derivative of *l*-, *dl*-, and *d*-tryptophane, respectively (equivalent to 0.2 per cent of tryptophane), and a fourth pair, 0.492 per cent of acetyl-*dl*-tryptophane. Of the second litter, one pair received acetyl-*d*-tryptophane; a second, acetyl-*l*-tryptophane; and a third, acetyl-*dl*-tryptophane, all to the extent of 0.123 per cent of the diet. These were controlled by pairs on *l*-tryptophane at 0.1 per cent (an equivalent amount) and at 0.05 per cent levels, a single animal on 0.15 per cent *l*-tryptophane, and a single animal on the unsupplemented tryptophane-deficient diet. The results

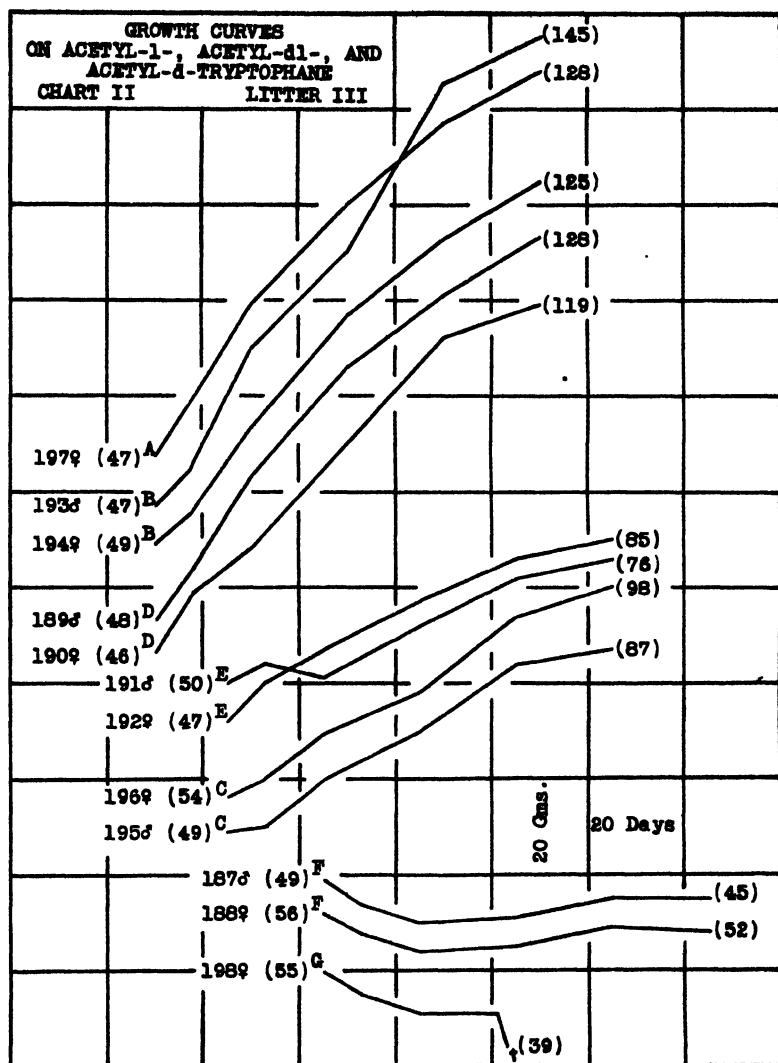


CHART II. A indicates that 0.15 per cent *l*-tryptophane was included in the diet; B, 0.10 per cent *l*-tryptophane; C, 0.05 per cent *l*-tryptophane; D, 0.123 per cent acetyl-*l*-tryptophane; E, 0.123 per cent acetyl-*dl*-tryptophane; F, 0.123 per cent acetyl-*d*-tryptophane; G, no tryptophane. The initial and final weights of the rats are given in parentheses. The dagger indicates the death of the animal.



obtained with both litters were essentially the same, although differences between the weight increments on acetyl-*l*- and on acetyl-*dl*-tryptophane were more marked at levels of 0.123 per cent than at the higher (0.246 per cent) levels. For economy of space only the growth curves and food consumption data for the second series are presented (Chart II and Table II). The data clearly confirm the conclusion of du Vigneaud, Sealock, and Van Etten (1932) that acetylation does not impair the utilization of

TABLE II  
*Food Consumption and Body Weight Changes*

Rat No. and sex	Average daily		Total change in weight	Tryptophane supplement
	Change in weight	Food consumption		
	gm.	gm.	gm.	per cent
197 ♀	+1.01	5.0	+81	0.15, <i>l</i> -
193 ♂	+1.23	5.6	+98	0.1, "
194 ♀	+0.95	5.3	+76	0.1, "
189 ♂	+1.00	4.9	+80	0.123, acetyl- <i>l</i> -
190 ♀	+0.91	4.7	+73	0.123, "
191 ♂	+0.32	4.1	+28	0.123, acetyl- <i>dl</i> -
192 ♀	+0.48	4.3	+38	0.123, "
195 ♂	+0.48	4.3	+38	0.05, <i>l</i> -
196 ♀	+0.55	4.3	+44	0.05, "
187 ♂	-0.05	2.7	-4	0.123, acetyl- <i>d</i> -
188 ♀	-0.05	3.0	-4	0.123, "
198 ♀ *	-0.42	3.2	-16	None

\* Rats on deficient diets of this nature usually lose rapidly at first and then maintain weight at a lower level. The early death of this rat accounts for the high average daily weight loss. See Chart II.

*l*-tryptophane, but does render *d*-tryptophane unfit for supporting growth. Acetyl-*dl*-tryptophane, in amount equivalent to 0.1 per cent of tryptophane, produced growth equivalent to that obtained on 0.05 per cent of tryptophane. The greater differences between weight increments on 0.1 per cent and 0.05 per cent of tryptophane (or its effective acetylated equivalent) than between 0.2 per cent and 0.1 per cent of tryptophane, emphasize the importance of employing only optimum or suboptimum amounts of the effective component in tests of this nature (Berg and Pot-

gieter, 1931-32). Unless this precaution is observed, individual differences in growth may obscure any attempt at quantitative evaluation.

*Kynurenic Acid Studies*—The technique of testing the compounds has been previously detailed (Berg, 1931). Essentially, it consists in administering the substance in question to rabbits (either orally or subcutaneously), collecting the 24 hour urine following, isolating the kynurenic acid by the Capaldi (1897) procedure, and washing this precipitate with water-saturated *n*-butyl alcohol to remove admixed tryptophane or acetyltryptophane. During the experimental period the rabbits were housed in individual metabolism cages, given water and oats *ad libitum*, and daily allotments of alfalfa hay and carrots. The compounds were administered every 3rd day. All 24 hour urines, whether collected on experimental or on intervening days, were analyzed according to the routine procedure.

Table III summarizes the data obtained after administration of *d*-, *l*-, and *dl*-tryptophane, either subcutaneously or by stomach tube. The amount of precipitate obtained after subcutaneous injection of *d*-tryptophane was not appreciably greater than that during control periods. The small amount of racemization of the *d*-tryptophane occurring during its isolation from its acetyl derivative (Berg, 1933) might readily account for the slight increase over the excretion on control days, if any accounting is necessary. The precipitate following *per os* administration, though still of doubtful significance, was found to be a trifle greater than in control periods. Unfortunately the output was not sufficient to permit testing for kynurenic acid with any degree of certainty. After *per os* administration of *d*-tryptophane, also, an appreciable precipitate, as yet unidentified but readily soluble in the butyl alcohol, was obtained. The extra washings required to leach out this contaminant completely may have removed not more than 7 additional mg. of kynurenic acid, if this amount were present. Any conversion of *d*-tryptophane to kynurenic acid under the conditions of these experiments was, indeed, exceedingly slight.

There are several factors which may contribute toward explaining why, in the rabbit, *d*-tryptophane produces no appreciable amount of kynurenic acid, although it supports growth in the rat. The difference in species may play some part, although both the

TABLE III

*Kynurenic Acid Elimination Following Administration of l-, dl-, and d-Tryptophane*

Day	Kynurenic acid precipitate after washing with 5 cc. butyl alcohol		Modification of tryptophane administered as sodium salt
	Rabbit 3 ♂, 2.2 kilos	Rabbit 4 ♂, 2.3 kilos	
	gm.	gm.	
1-2	0.0154	0.0063	
3	0.2446	*0.3614	1 gm. l-, 1 dose
4-5	0.0153	0.0128	
6	0.1262	0.1640	1 " dl-, 2 doses
7-8	0.0094	0.0075	
9	0.1523	0.0689	1 " " 4 "
10-11	0.0017	0.0020	
12	0.1057	0.1211	0.5 " l-, 2 "
13-14	0.0027	0.0157	
15	0.2261	0.2852	1 " " 2 "
16	0.0042	0.0028	
	Rabbit 11 ♂, 2.1 kilos	Rabbit 12 ♂, 2.5 kilos	
1-2	0.0030	0.0017	
3	0.1890	0.1666	0.5 gm. l-, 2 doses
4-5	0.0060	0.0036	
6	0.0048	0.0070	1 " d-, 2 "
7-8	0.0024	0.0040	
9	0.2061	0.2423	1 " dl-, 2 "
10-11	0.0123	0.0157	
12	0.3107	0.2527	1 " l-, 2 "
13	0.0054	0.0039	
	Rabbit 7 ♂, 2.3 kilos	Rabbit 8 ♂, 2.3 kilos	
1-3	0.0022	0.0032	
4	0.2576	0.2531	1 gm. l-*
5-6	0.0012	0.0028	
7	0.1425	0.1798	1 " dl-*
8-9	0.0012	0.0018	
10	0.1196	0.1602	0.5 " l-*
11-12	0.0062	0.0018	
13	0.0147†	0.0098†	1 " d-*
14	0.0116	0.0130	

The values for days 1 to 2, etc., are the averages per day.

\* All of the administrations thus indicated were by stomach tube in one dose; all others were by subcutaneous injection.

† An extra portion of butyl alcohol was used for washing out the contaminants in these precipitates.

rabbit and the rat excrete kynurenic acid. The 1 gm. dose administered to the rabbit is massive, even when differences in body weight are considered, in comparison with the average maximum daily intake of 10 to 11 mg. of *d*-tryptophane by the rat in the preceding growth studies. Qualitative evidence was, in fact, obtained (by Hopkins-Cole test) that whereas, after *l*-tryptophane injection in the rabbit, no indole substances appeared in the urine, they were present after *dl*-tryptophane and *d*-tryptophane injection. This obviously indicates that the unnatural isomer is the less readily utilized by the body, and therefore, the more readily excreted. It seems fair to assume, however, in view of work on other amino acids previously cited (see Berg and Potgieter, 1931-32), that that part of the unnatural dextro isomer which escapes excretion may undergo deaminization in the body, presumably to form indolepyruvic acid. One may assume from the more ready excretion of *d*-tryptophane that this modification undergoes a comparatively slower oxidative deaminization than does the *l*-tryptophane. If this assumption be correct, then the massive dose might, for the major part, escape conversion, whereas the 10 or 11 mg. consumed over a 24 hour period in the growth studies might undergo transformation into the ketonic acid and thence to the levo isomer, if conversion into *l*-tryptophane is an obligative step. Indolepyruvic acid has been shown to replace tryptophane effectively in promoting growth (Jackson, 1929; Berg, Rose, and Marvel, 1929-30, *b*). As a precursor of kynurenic acid it is very much less effective than *l*-tryptophane. Ellinger and Matsuoka (1920), in a series of tests, found the average yield of kynurenic acid from indolepyruvic acid to be 28 per cent of that from an equivalent amount of *l*-tryptophane. A study of their data reveals that the output of kynurenic acid after one of the tryptophane administrations was unusually low, and that the above figure may therefore be high. Any *d*-tryptophane undergoing conversion to indolepyruvic acid would thus be inefficiently converted into kynurenic acid. The slight increment in apparent kynurenic acid output following *d*-tryptophane administration *per os*, over that after its injection subcutaneously, may be a reflection of a slower absorption rate which would permit a greater degree of deaminization and consequent conversion into indolepyruvic acid. We are investigating these aspects of the case further. In any

TABLE IV

*Kynurenic Acid Elimination Following Administration of Acetyl-l-, Acetyl-dl-, and Acetyl-d-Tryptophane*

Day	Kynurenic acid precipitate after washing with butyl alcohol		Tryptophane or derivative administered as sodium salt
	Rabbit 1 ♂, 2.6 kilos	Rabbit 2 ♂, 2.1 kilos	
	gm.	gm.	
1-2	0 0068	0 0041	
3	0 0995	0 1527	1.23 gm. acetyl-l-, 2 doses
4-5	0 0067	0 0057	
6	0 0612	0 1310	1.23 " " 2 "
7-8	0 0069	0 0075	
9	0 0424*	0 0646*	1.23 " acetyl-dl-, 2 "
10-11	0 0032	0 0006	
12	0 0496	0 0854	0.615 " acetyl-l-, 2 "
13-14	0 0010		
15	0 2751		1 gm. l-, 2 doses
16-17	0 0026		
	Rabbit 9 ♂, 2.3 kilos	Rabbit 10 ♂, 2.6 kilos	
	gm.	gm.	
1-3	0 0028	0 0018	
4	0 4123	0 3026	1 gm. l-†
5-6	0 0015	0 0023	
7	0 1541	0 0533	1.23 gm. acetyl-l-†
8-9	0 0014	0 0038	
10	0 0401	0 0172	1.23 " acetyl-dl-†
11-12	0 0051	0 0049	
13	0 0584	0 0506	0.615 " acetyl-l-†
14-16	0 0039	0 0037	
17	0 0010*	0 0043*	1.23 " acetyl-d-†
18-19	0 0019	0 0032	
20	0 1087*	0 1318	1.23 " acetyl-l-, 2 doses
21-22	0 0048	0 0017	
23	0 0099*	0 0140*	1.23 " acetyl-d-, 2 "
24-25	0 0083	0 0106	
26	0 0925*	0 0866*	1.23 " acetyl-dl-, 2 "
27	0 0039	0 0047	

The values for days 1 to 2, etc., are the averages per day.

\* In the cases indicated, more than 5 cc. of the butyl alcohol were required for the washing.

† All of the administrations thus indicated were by stomach tube in one dose; all others were by subcutaneous injection.

event, it seems clear that *d*-tryptophane cannot be converted directly into kynurenic acid. A survey of Table III further indicates that, on the average, 1 gm. of *dl*-tryptophane yields approximately one-half (0.1603 gm.) of the kynurenic acid excreted after the administration of 1 gm. of *l*-tryptophane (0.2739 gm.) or an output approximately equivalent to that resulting from 0.5 gm. of *l*-tryptophane (0.1437 gm.).

Table IV records the kynurenic acid excretion following the administration, either by stomach tube or by subcutaneous injection, of acetyl-*d*-, acetyl-*dl*-, and acetyl-*l*-tryptophane. In every instance, even after subcutaneous injection, the urines responded positively to the Hopkins-Cole test. After acetyl-*d*- and acetyl-*dl*-tryptophane administration, a considerable portion of these derivatives was isolated along with the kynurenic acid; in a few instances, following acetyl-*l*-tryptophane administration, this isomer also was excreted in appreciable amounts. The kynurenic acid was always freed of these contaminants by washing them out with butyl alcohol. To judge from a few isolation experiments not recorded here, in which special care was taken to avoid racemization during the separation of the excreted derivative, acetyl-*dl*-tryptophane does not undergo ready resolution in the body. The product isolated, after the administration *per os* of 2 gm. of acetyl-*dl*-tryptophane, showed a specific rotation approximately 5 per cent of that of acetyl-*d*-tryptophane. Observation of the kynurenic acid output on the three isomeric modifications of acetyltryptophane indicates that acetyl-*d*-tryptophane cannot be utilized for kynurenic acid production. The average yield of this product from 1.23 gm. of acetyl-*dl*-tryptophane (0.0572 gm.) was approximately half of that from the same amount of acetyl-*l*-tryptophane (0.1116 gm.), or an output approximately equivalent to that resulting from 0.615 gm. of acetyl-*l*-tryptophane (0.0610 gm.). The average amount of kynurenic acid produced from 1.23 gm. of acetyl-*l*-tryptophane was, in turn, much less than that from an equivalent weight (1 gm.) of the free *l*-tryptophane (0.3300 gm.). Hence, our previous conclusions, from work on acetyltryptophane (Berg, 1931) now known to have been racemic, are essentially correct.

## SUMMARY

A comparison of growth rates and efficiency quotients obtained on *d*- and *l*-tryptophane indicates that these isomers are equally effective in supporting growth in the rat. On the other hand, the apparent kynurenic acid excretion in the rabbit was so slight after *d*-tryptophane administration (either *per os* or subcutaneously) as to indicate no appreciable production from this modification. The yield from *dl*-tryptophane was found to be approximately half of that from an equal weight of *l*-tryptophane.

Acetyl-*d*-tryptophane was not utilized either for growth or for kynurenic acid production. Acetylation of *l*-tryptophane does not appreciably affect its growth-promoting ability, but does reduce considerably its convertibility into kynurenic acid.

The data on growth emphasize the importance of avoiding the use of higher than optimum levels of an essential component in comparative tests.

Possible explanations for the difference in growth and kynurenic acid responses have been suggested.

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## N-METHYL URIDINE AND ITS BEARING ON THE STRUCTURE OF URIDINE

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(Received for publication, December 27, 1933)

The generally accepted allocation of the ribose residue to position (3) of the base in the case of uridine and cytidine is based on theoretical considerations and on experimental evidence of an indirect nature. Even in the case of the synthetic glucosido-uracil of Hilbert and Johnson<sup>1</sup> the conclusion as to the position of the glucose residue rests on analogy.<sup>2</sup>

Direct experimental evidence is now furnished substantiating the generally accepted view.

Crystalline trityl uridine was prepared by condensing uridine with triphenylmethyl chloride in the presence of dry pyridine. The resulting product, after careful recrystallization, was found to possess properties quite different from those recorded by Brederbeck.<sup>3</sup> Whereas his material was an amorphous, hygroscopic substance which melted with foaming at 115–116° and had  $[\alpha]_D^{19} = +11.0^\circ$  (in chloroform), our trityl uridine is crystalline and non-hygroscopic, and melts sharply at 200° without foaming. It is not sufficiently soluble in chloroform to permit a determination of its specific rotation in that solvent at the concentration recorded by Brederbeck.

Pure trityl uridine was acetylated and the resulting diacetyl trityl uridine was treated with excess diazomethane in cold dry ether, thereby acquiring a methyl group (Table I).

It was shown that this methyl group is attached to the nitrogen

<sup>1</sup> Hilbert, G. E., and Johnson, T. B., *J. Am. Chem. Soc.*, **52**, 4489 (1930).

<sup>2</sup> A detailed discussion of the experimental and theoretical arguments are given in the monograph of Levene and Bass (Levene, P. A., and Bass, L. W., *Nucleic acids*, pp. 146–149 (1931)).

<sup>3</sup> Brederbeck, H., *Ber. chem. Ges.*, **65**, 1830 (1932).



atom at position (1) of the uracil residue since, on complete hydrolysis, 1-methyl uracil was obtained. This monomethyl uracil was identical with that synthesized from 1-methyl-2-ethylmercapto-6-oxypyrimidine.<sup>4</sup>

It therefore follows that in uridine position (3) in the uracil residue is linked to the glycosidic group of the ribose residue and that uridine is 3'-uracil ribofuranoside.<sup>5</sup>

The same result might have been obtained with triacetyl uridine. The diacetyl trityl derivative was utilized for the reason that the substance was immediately available, having been prepared for a

TABLE I  
*Comparison of Uridine and N-Methyl Uridine, and of Their Derivatives*

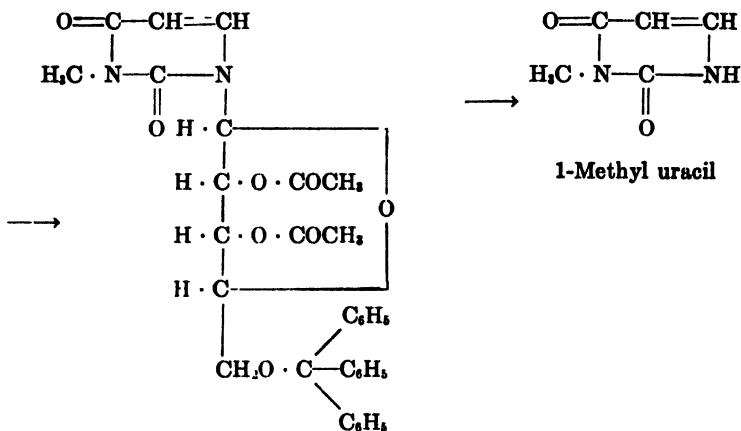
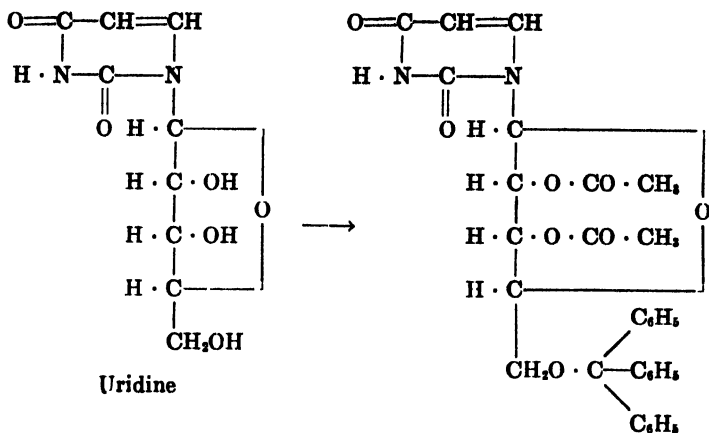
Diacetyl trityl uridine	Diacetyl trityl N-methyl uridine
Colorless glass [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +50.9° (in absolute methyl alcohol)	Colorless glass [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +55.3° (in absolute methyl alcohol)
Trityl uridine	Trityl N-methyl uridine
Colorless crystals; m.p. 200° [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +9.5° (in acetone)	Colorless crystals; m.p. 173-174° [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +17.1° (in acetone)
Uridine	N-Methyl uridine
Colorless crystals; m.p. 165° [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +4.0° (in water)	Colorless crystals; m.p. 108-110° [ $\alpha$ ] <sub>D</sub> <sup>20</sup> = +16.5° (in water)

different purpose.<sup>6</sup> Trityl uridine is represented in the accompanying formulæ as the 5-substituted derivative, though this structure has not hitherto been definitely proved. We have succeeded in isolating crystalline methylated trityl uridine by the methylation of trityl uridine and its structure will be discussed in a subsequent communication.

<sup>4</sup> Johnson, T. B., and Heyl, F. W., *Am. Chem. J.*, **37**, 628 (1907).

<sup>5</sup> Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **101**, 529 (1933).

<sup>6</sup> We had intended to employ this material for the synthesis of uridine-5-phosphoric acid, but this synthesis has now been accomplished through the phosphorylation of the beautifully crystalline monoacetone uridine we have recently prepared. The properties of these compounds and of crystalline methylated monoacetone uridine will be described in the near future.



Diacetyl trityl N-methyl uridine

## EXPERIMENTAL

*Preparation of Monotrityl Uridine*—A mixture of 10 gm. of dry, finely powdered uridine with 12.6 gm. of pure, dry triphenylmethyl chloride was dissolved in 120 cc. of dry, redistilled pyridine. The solution was allowed to stand overnight at room temperature, after which it was heated, under an air condenser closed by a calcium chloride tube, in a glycerol bath at 100° during 3 hours.

The solution was then cooled and poured into 1 liter of water, with vigorous stirring. The cloudy supernatant liquor was decanted from the gummy precipitate, filtered, and the filtrate discarded. The gummy material was well washed with water, and then dissolved in acetone, and the solution evaporated to dryness under diminished pressure.

It was freed from pyridine by heating twice with 200 cc. portions of boiling water, cooling, and discarding the water. The solid mass was now dissolved in 250 cc. of a mixture of acetone with absolute ethyl alcohol (1:1), the solution mixed with a little charcoal, and filtered. The filtrate was evaporated under diminished pressure to a hard mass which was dried by dissolving in absolute ethyl alcohol and again evaporating to dryness. The yield of crude material was practically quantitative.

The product was dissolved in 50 cc. of acetone and a little dry ether was cautiously added with shaking, whereupon crystallization rapidly set in. After standing overnight at room temperature the crystals were filtered off, washed with acetone and dry ether, and dried. The first crop of crystals weighed 9.5 gm. The mother liquor was evaporated to a colorless glass, weighing approximately 10.5 gm., which was fractionally crystallized.

After several recrystallizations the substance melted sharply at 200° without foaming. It was insoluble in cold or hot carbon tetrachloride, pentane, and water; insoluble in cold but sparingly soluble in hot benzene; slightly soluble in cold but more soluble in hot ethyl acetate and in dry ether; and quite soluble in the following solvents in the cold: absolute ethyl alcohol, absolute methyl alcohol, acetone, chloroform, pyridine, and glacial acetic acid.

It had the following composition.

5.015 mg. substance:	12.705 mg. CO <sub>2</sub> and 2.685 mg. H <sub>2</sub> O
100 " "	required 4.05 cc. 0.1 N HCl (Kjeldahl)
C <sub>23</sub> H <sub>26</sub> O <sub>6</sub> N <sub>2</sub> .	Calculated. C 69.11, H 5.39, N 5.76
	Found. " 69.09, " 5.99, " 5.67

The substance displayed the following specific rotations.

$$[\alpha]_D^{25} = \frac{+0.20^\circ \times 100}{2 \times 1.056} = +9.5^\circ \text{ (in acetone)}$$

$$[\alpha]_D^{25} = \frac{+0.39^\circ \times 100}{2 \times 1.036} = +18.8^\circ \text{ (in absolute methyl alcohol)}$$

*Preparation of Diacetyl Trityl Uridine*—10 gm. of dry, recrystallized trityl uridine (having  $[\alpha]_D^{28} = +9.3^\circ$  (in acetone); m.p. =  $200^\circ$ ) were dissolved in 60 cc. of dry, redistilled pyridine and 40 cc. of acetic anhydride were added. After standing at room temperature overnight, the solution was poured into 800 cc. of cold water with vigorous stirring.

The white powdery precipitate was filtered off and washed several times with distilled water. It was then dissolved in acetone and the solution evaporated to dryness under diminished pressure. The glassy product was dissolved in absolute alcohol, a little charcoal added, and the mixture filtered. The filtrate was evaporated to dryness giving a practically quantitative yield of a colorless, flaky glass which was insoluble in cold or hot pentane or water, but readily soluble in the following solvents in the cold: absolute ethyl alcohol, absolute methyl alcohol, dry ether, acetone, chloroform, carbon tetrachloride, benzene, pyridine, ethyl acetate, and glacial acetic acid.

For analysis, the flaky, glass-like substance was dried in the vacuum oven at  $80^\circ$  during 48 hours. It had the following composition.

4.670 mg. substance: 11.590 mg.  $\text{CO}_2$  and 2.305 mg.  $\text{H}_2\text{O}$

6.165 " : 0.272 cc.  $\text{N}_2$  (751 mm. at  $28^\circ$ )

$\text{C}_{22}\text{H}_{24}\text{O}_6\text{N}_2$ . Calculated. C 67.33, H 5.30, N 4.91

Found. " 67.67, " 5.52, " 4.94

100 mg. substance required 3.60 cc. 0.1 N NaOH (alkaline hydrolysis)

$\text{C}_{22}\text{H}_{24}\text{O}_6\text{N}_2 \cdot (\text{COCH}_3)_2$ . Calculated.  $\text{COCH}_3$  15.09

Found. " 15.48

It had the following specific rotation.

$$[\alpha]_D^{28} = \frac{+1.01^\circ \times 100}{2 \times 0.992} = +50.9^\circ \text{ (in absolute methyl alcohol)}$$

*Methylation of Diacetyl Trityl Uridine by Means of Diazomethane*—12 gm. of dry diacetyl trityl uridine were dissolved in dry ether and the solution cooled to  $0^\circ$ , whereupon some of the substance settled out as a colorless syrup. To this was added an ice-cold solution of about 3 gm. of diazomethane in dry ether. On shaking at  $0^\circ$  for a few minutes the diacetyl trityl uridine dissolved completely, giving a clear solution colored yellow by the excess diazomethane.

After standing overnight at room temperature with the exclusion of atmospheric moisture, the pale yellow solution was evaporated to dryness under diminished pressure to give the theoretical yield of a pale yellow, glass-like substance. It was dried by dissolving in benzene and evaporating to dryness, the operation being repeated once more with benzene and then twice with dry ether. The colorless, flaky, glass-like product was dried in the vacuum oven at 60° during 48 hours.

It was insoluble in cold or hot pentane and water, but readily soluble in the following solvents in the cold: absolute ethyl alcohol, absolute methyl alcohol, dry ether, acetone, chloroform, carbon tetrachloride, benzene, pyridine, ethyl acetate, and glacial acetic acid.

It had the following composition.

4.956 mg. substance: 12.380 mg. CO<sub>2</sub> and 2.455 mg. H<sub>2</sub>O

6.240 " " : 0.260 cc. N<sub>2</sub> (766 mm. at 23°)

C<sub>11</sub>H<sub>12</sub>O<sub>4</sub>N<sub>2</sub>. Calculated. C 67.77, H 5.52, N 4.80

Found. " 68.12, " 5.54, " 4.84

100 mg. substance required 3.33 cc. 0.1 N NaOH (alkaline hydrolysis)

C<sub>11</sub>H<sub>12</sub>O<sub>4</sub>N<sub>2</sub>·(COCH<sub>3</sub>)<sub>2</sub>. Calculated. COCH<sub>3</sub> 14.72

Found. " 14.33

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+1.14^\circ \times 100}{2 \times 1.030} = +55.3^\circ \text{ (in absolute methyl alcohol)}$$

*Deacetylation of Diacetyl Trityl N-Methyl Uridine*—6.5 gm. of dry, flaky diacetyl trityl N-methyl uridine were dissolved in 165 cc. of absolute methyl alcohol and the solution cooled to 0°. 2.5 cc. of a 0.25 M solution of barium methylate in absolute methyl alcohol were added and the solution kept at 0° during 45 hours.

The solution was then saturated in the cold with carbon dioxide and 35 cc. of cold distilled water were added. The solution was again saturated with carbon dioxide and was then heated until gently boiling. A little charcoal was added, the mixture filtered, and the filtrate evaporated to dryness under diminished pressure at 30°, giving the theoretical yield of a colorless glass. On dissolving in 10 cc. of warm acetone and adding about 30 cc. of dry ether (to faint opalescence) the substance crystallized. It was recrystallized from a mixture of acetone and dry ether giving beautiful

colorless crystals, with a melting point of 173–174°. On admixture with trityl uridine (m.p. 200°) the mixture softened at 166–169° and melted at 170–175°. It was soluble in the cold in the following solvents: absolute ethyl alcohol, absolute methyl alcohol, acetone, chloroform, benzene, pyridine, ethyl acetate, glacial acetic acid; insoluble in cold or hot carbon tetrachloride, pentane, and water; sparingly soluble in hot dry ether.

It had the following composition.

4.800 mg. substance: 12.240 mg. CO<sub>2</sub> and 2.440 mg. H<sub>2</sub>O

6.422 " " : 0.314 cc. N<sub>2</sub> (761 mm. at 18°)

C<sub>25</sub>H<sub>25</sub>O<sub>6</sub>N<sub>3</sub>. Calculated. C 69.57, H 5.64, N 5.60

Found. " 69.55, " 5.68, " 5.74

It had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.36^\circ \times 100}{2 \times 1.052} = +17.1^\circ \text{ (in acetone)}$$

*Preparation of N-Methyl Uridine*—13 gm. of diacetyl trityl N-methyl uridine were dissolved in 100 cc. of absolute methyl alcohol. To the solution were added 100 cc. of absolute methyl alcohol containing 1 gm. of dry hydrogen chloride and the resulting solution was gently boiled under reflux during 10 minutes. The solution was then kept overnight at room temperature.

The clear, pale yellow solution was now rendered neutral by addition of dry silver carbonate, the mixture filtered, and the silver salts extracted with boiling methyl alcohol under reflux. The filtrate and extracts were combined and evaporated to dryness under diminished pressure, giving a pale yellow mass which was partly gummy and partly crystalline.

It was stirred with water and benzene until all the material had dissolved. The two layers were separated and the aqueous layer shaken twice with fresh portions of benzene. The aqueous layer was evaporated to dryness under diminished pressure to a colorless glass (weight, 5 gm.) which was dried by dissolving in absolute methyl alcohol and reevaporating to dryness. It was obtained crystalline by dissolving in 10 cc. of warm absolute methyl alcohol, adding 10 cc. of ethyl acetate and then dry ether to faint opalescence; whereupon it commenced crystallizing immediately in long rectangular plates with a melting point of 108–110°. It was

insoluble in cold or hot dry ether, carbon tetrachloride, benzene, or pentane; insoluble in cold but sparingly soluble in hot chloroform; fairly soluble in cold but more soluble in hot ethyl acetate; and quite soluble in the following solvents in the cold: absolute ethyl alcohol, absolute methyl alcohol, acetone, pyridine, glacial acetic acid, and water.

It had the following composition.

4.547 mg. substance:	7.780 mg. CO <sub>2</sub> and 2.255 mg. H <sub>2</sub> O
6.338 " " :	0.590 cc. N <sub>2</sub> (754 mm. at 22°)
C <sub>10</sub> H <sub>14</sub> O <sub>6</sub> N <sub>2</sub> . Calculated.	C 46.49, H 5.47, N 10.85
Found.	" 46.63, " 5.52, " 10.68

Its rotation was

$$[\alpha]_D^{25} = \frac{+0.33^\circ \times 100}{2 \times 1.000} = +16.5^\circ \text{ (in water)}$$

*Hydrolysis of N-Methyl Uridine*—2 gm. of crystalline N-methyl uridine were dissolved in 60 cc. of 10 per cent sulfuric acid and the solution heated in a sealed tube in an oil bath at 125° during 4 hours. The resulting solution was light brown in color and a small amount of melanin material had separated out.

The mixture was filtered and to the filtrate barium carbonate was added until most of the sulfuric acid had been removed. The sulfuric acid was then removed quantitatively by cautious addition of cold, saturated barium hydroxide solution. The barium sulfate was removed by centrifuging and washed three times by stirring with water and centrifuging. The solution and washings were combined and evaporated to dryness under diminished pressure giving a light brown gum which partly crystallized on standing.

It was dissolved in absolute ethyl alcohol and filtered from a trace of barium sulfate. Benzene was added to the filtrate to faint opalescence. On standing, a brown gum separated out. The clear, pale yellow, supernatant liquor was decanted and on allowing to evaporate slowly the material crystallized: melting point 174–175° alone or when admixed with an authentic specimen of 1-methyl uracil (m.p. 174–175°) prepared from 1-methyl-2-ethyl-mercapto-6-oxypyrimidine. It was soluble in the cold in the following solvents: absolute ethyl alcohol, absolute methyl alco-

hol, dry ether, acetone, chloroform, pyridine, ethyl acetate, glacial acetic acid, and water; insoluble in cold or hot pentane; insoluble in cold carbon tetrachloride, sparingly soluble in the hot; insoluble in cold benzene, readily soluble in the hot.

It had the following composition.

4.760 mg. substance: 8.290 mg.  $\text{CO}_2$  and 2.000 mg.  $\text{H}_2\text{O}$

3.890 " " : 0.736 cc.  $\text{N}_2$  (767 mm. at  $22^\circ$ )

$\text{C}_8\text{H}_8\text{O}_2\text{N}_2$ . Calculated. C 47.58, H 4.80, N 22.22

Found. " 47.50, " 4.70, " 22.09





## THE SPECIFIC GRAVITY OF SYNTHETIC SOLUTIONS OF SERUM ALBUMIN AND SERUM GLOBULIN

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Moore and Van Slyke (1) find the specific gravity of plasma to be a more useful measure of total plasma protein in nephritis than the refractive index, the physical property previously most widely employed in this connection. The effects of varying salt, glucose, and non-protein nitrogen contents presumably prevent accurate protein determinations by the specific gravity method, but are not sufficient to interfere with obtaining valuable practical results.

With regard to the future application of the method, the question arises as to how nearly the albumin and globulin fractions exert equivalent influences upon the specific gravity of plasma, since, if these are not nearly the same, the indicated total protein content in any case will depend upon the albumin-globulin ratio. The first step in an answer to this question should be obtainable from the results of measurements of the specific gravities of synthetic solutions of serum albumin, serum globulin, and their mixtures, which have accordingly been carried out.

Fresh beef blood was allowed to clot, and the globulin fraction precipitated from the resulting serum by half saturation with ammonium sulfate. The albumin was precipitated from the globulin filtrate by saturation with ammonium sulfate. Both fractions were then dialyzed in cellophane sacks until negligible tests for sulfate were obtained. During the dialysis and in subsequent procedures, the solutions were protected from bacterial action by the presence of crystals of thymol. Precipitated globulin was put back in solution by the addition of a minimum quantity of sodium chloride. Both solutions were adjusted to approximately 12 per cent protein content by evaporation in a current of warm air, and their total protein contents then determined by drying weighed portions to constant weight at 105°

and correcting for the salt content of the globulin solution. They were next adjusted to 0.9 per cent sodium chloride content and to pH 7.3 to 7.5 by the use of a simple colorimetric method analogous to one used by Cullen (2) for the determination of the pH of plasma. When necessary, chloride determinations were carried out with Wilson and Ball's modification of Van Slyke's method for plasma chlorides (3). With the albumin and globulin solutions

TABLE I

*Specific Gravities at 25° of Synthetic Solutions of Beef Serum Albumin and Serum Globulin Containing 0.9 Per Cent Sodium Chloride and Adjusted to pH 7.3 to 7.5*

Solution No.	Total protein content	Albumin-globulin ratio	Sp. gr.	Solution No.	Total protein content	Albumin-globulin ratio	Sp. gr.
	<i>per cent</i>				<i>per cent</i>		
1	12.31	100:0	1.0405	16	12.12	40:60	1.0397
2	10.00	100:0	1.0338	17	10.35	40:60	1.0354
3	8.00	100:0	1.0283	18	8.28	40:60	1.0295
4	6.00	100:0	1.0228	19	6.22	40:60	1.0241
5	4.00	100:0	1.0177	20	4.14	40:60	1.0177
6	2.00	100:0	1.0118	21	2.07	40:60	1.0120
7	12.24	80:20	1.0399	22	12.06	20:80	1.0399
8	10.00	80:20	1.0338	23	10.00	20:80	1.0340
9	6.00	80:20	1.0228	24	8.00	20:80	1.0285
10	4.00	80:20	1.0175	25	6.00	20:80	1.0230
11	2.00	80:20	1.0124	26	4.00	20:80	1.0177
12	9.85	60:40	1.0342	27	2.00	20:80	1.0120
13	7.88	60:40	1.0289	28	11.60	0:100	1.0384
14	5.93	60:40	1.0230	29	9.67	0:100	1.0331
15	2.80	60:40	1.0139	30	7.74	0:100	1.0278
				31	5.78	0:100	1.0222
				32	3.87	0:100	1.0177
				33	2.00	0:100	1.0122

so prepared as stock solutions, it was a simple matter to prepare a series of solutions of pH 7.3 to 7.5 with 0.9 per cent sodium chloride content, and varying in total protein content from 1 to 12 per cent and in albumin-globulin ratio as desired.

Relative viscosity determinations were made on the 100 per cent albumin and 100 per cent globulin fraction series with a 1 cc. Ostwald viscometer at 25°. The viscosity values in each case were plotted against the respective concentrations. The curves

obtained were the typically contrasting curves of albumin and globulin (4), thus proving the separation of the original serum proteins into distinct albumin and globulin fractions.

The specific gravities of all solutions were determined at 25° with a 0.5 cc. Nicol type pycnometer, a water bath thermostat, and an analytical balance. Wherever mentioned in this paper, the specific gravity of a solution refers to the ratio of the weight of a given volume of that solution at 25° to the weight of an equal volume of distilled water at the same temperature. The results are shown in Table I.

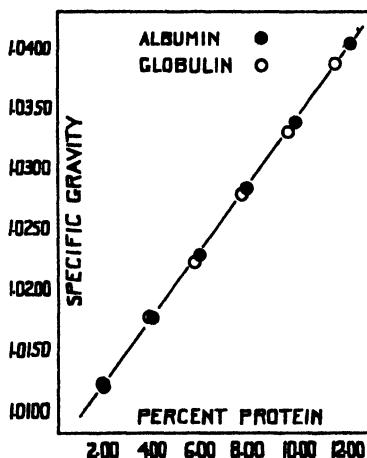


FIG. 1. The specific gravity values for the 100 per cent albumin and 100 per cent globulin fractions plotted against the respective total protein contents.

When the specific gravity is plotted against the total protein concentration, the points for all albumin-globulin ratios are found to be distributed uniformly about a single straight line which is defined by the equation,  $\text{specific gravity} = 1.0067 + 0.00273 C$ , where 1.0067 is the specific gravity of 0.9 per cent sodium chloride solution and  $C$  is the total protein concentration in per cent. The maximum deviation of any of the values from the line, expressed in specific gravity units, is 0.0006 gm. per cc. and the average deviation of all the values, 0.0002 gm. per cc., the latter being with-

in the estimated limits of experimental error. The points for the 100 per cent albumin and 100 per cent globulin fractions are shown in Fig. 1.

#### SUMMARY

The general conclusion is that, under the experimental conditions described, beef serum albumin and serum globulin exert effects upon the specific gravities of their synthetic solutions which are identical within the limits of experimental error of the methods usually employed for the accurate determination of specific gravity values. This fact is of importance for the reasons discussed in the first part of the paper. It is also of interest from the point of view of the general knowledge of protein solutions.

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# A CONTRIBUTION TO THE CHEMISTRY OF LACTO-BACILLUS ACIDOPHILUS

## II. COMPOSITION OF THE NEUTRAL FAT\*

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(Received for publication, December 20, 1933)

The work reported in this paper is a continuation of an investigation on the chemical composition of the alcohol-ether-soluble constituents present in *Lactobacillus acidophilus*. This organism has attained during the past few years considerable importance in connection with implantation in the human intestinal tract for the relief of various intestinal disorders. It has been shown by the work of Rettger and his students (1) that *Lactobacillus acidophilus* can be implanted readily and that such implantation exerts in general a beneficial effect. These observations have been confirmed by numerous other investigations (2), and at the present time the *Lactobacillus* is grown extensively on a commercial scale for the preparation of acidophilus milk and other products.

Since no chemical investigations of this organism had been reported, it seemed to us worth while to begin some work on this subject. We have already described an optically active dihydroxystearic acid which occurred free in the fat extracted from the *Lactobacillus* (3). It is perhaps of significance in connection with the physiology of this acidophilic organism that it produces a large amount of free fatty acids. The total lipids were found to contain about 28 per cent of free acids.

As indicated in our first report (3) the *Lactobacillus* was grown in a natural medium. It is very probable, therefore, that some of the ether-soluble constituents contained in the medium might be adsorbed on the surface of the bacteria and thus complicate

\* The data are taken from the dissertation submitted by J. A. Crowder to the faculty of the Graduate School, Yale University, 1933, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

the interpretation of the results of the chemical analyses. We have found, for instance, that the neutral fat contained an appreciable quantity of cholesterol. The question whether the cholesterol was derived from the skim milk used in the medium or from the bacteria cannot be settled until an examination has been made of the bacillus when grown in a medium which is free from cholesterol.

The neutral fat which had been separated from the free fatty acids and phosphatide amounted to 35 per cent of the total lipids. The present report deals with the composition of this fraction and the results indicate that the fat is a mixture of glycerides of lauric, myristic, palmitic, stearic, and oleic acids.

#### EXPERIMENTAL

The X type of *Lactobacillus acidophilus* used in this investigation was grown in the Mulford Biological Laboratories, Sharp and Dohme. The composition of the medium was described in the former publication (3). The bacterial mass was collected in supercentrifuges, washed with water, and stirred into denatured alcohol (9 parts of ethyl alcohol and 1 part of methyl alcohol). For the present work we received four 5 gallon bottles of the bacterial suspension, each bottle containing 10 liters of the denatured alcohol, to which had been added sufficient moist bacteria to fill the container.

Throughout this work we adhered as closely as possible to the methods used in this laboratory in the investigations of the lipids of tubercle bacilli (4). Air was always displaced by carbon dioxide or nitrogen and freshly purified solvents were used.

*Extraction of Lipids*—In order to facilitate filtration of the bacterial suspensions, each bottle received was mixed thoroughly with 4 liters of alcohol, after which the bacteria were filtered off on large Buchner funnels. The cells were next treated with 12 liters of alcohol with occasional shaking for 3 days, filtered, and washed with alcohol. This treatment was followed by two extractions for 3 days with 12 liters of a mixture containing equal parts of alcohol and ether. The extracts were combined and worked up as described below.

The cell residues, after two further extractions with chloroform, were washed free of chloroform with alcohol and dried in a vacuum drier. The dried bacterial residue weighed 5.5 kilos.

The combined alcohol-ether extracts were concentrated in the usual manner (4) until most of the solvents were removed and the crude fat was extracted with ether. The ethereal extract was dried with sodium sulfate, filtered through a Chamberland candle, and evaporated to dryness. The residue formed a dark solid mass which weighed 397.4 gm.

The chloroform extracts and washings were combined and evaporated to dryness. The residue thus obtained was a dark brown mass which weighed 21 gm. and was not completely soluble in ether. This fraction has not been analyzed. The result indicates that practically all of the lipid material is extracted from the acidophilus bacillus with alcohol and ether, and that the organism does not contain any appreciable quantity of wax-like material.

The total lipid constituents obtained from the bacillus amounted therefore to 418.4 gm., which equals about 7 per cent.

*Separation of Lipid Fractions*—The crude fat from the alcohol-ether extracts was treated with 2 liters of absolute ether. A cloudy solution resulted from which a flocculent precipitate separated. The precipitate was filtered off, washed with absolute ether, and dried. The substance was a red-colored amorphous powder and the total yield amounted to 13.6 gm., or 3.4 per cent of the crude fat. From a smaller portion of the fat some of the same substance had been isolated, as described in a former paper (3) and identified as dihydroxystearic acid.

The ethereal solution was mixed with 3 liters of acetone which caused the precipitation of the crude phosphatide as a sticky mass. After the mixture had been cooled, the clear supernatant solution was decanted and the insoluble portion was washed with acetone.

The ether-acetone solution was concentrated to dryness and yielded 242 gm. of crude acetone-soluble fat. Since the latter was found to contain nearly 40 per cent of free fatty acids, it was decided to separate the acids from the neutral fat. The material was dissolved in ether and extracted four times with 2 per cent potassium hydroxide. The ethereal solution on concentration to dryness gave 140.1 gm. of a light yellow-colored neutral fat. From the alkaline solution after acidification and extraction with ether there were obtained 96.7 gm. of a dark colored semisolid mass consisting of free fatty acids. This fraction has not been analyzed.



**Purification of Phosphatide**—The crude phosphatide was dissolved in 200 cc. of absolute ether and the solution, after it had been centrifuged to remove a small amount of insoluble material, was precipitated with acetone. The precipitate was again treated with 200 cc. of absolute ether and a trace of insoluble matter was removed by centrifugation. The ether-insoluble substance weighed only 1.2 gm. and was not further examined. The ethereal solution was again precipitated with acetone and the phosphatide, which was now completely soluble in ether, was reprecipitated eight times in the same manner; the last precipitation was carried out by pouring the ethereal solution into cold acetone. The purified phosphatide was obtained as an amorphous powder of light brown color and it weighed 125.1 gm.

For analysis the substance was dried to constant weight at 60° *in vacuo* over phosphorus pentoxide.

0.5011, 0.6130 gm. substance: 0.0262, 0.0308 gm.  $Mg_2P_2O_7$ ,

1.2014 gm. substance (Kjeldahl): 10.40 cc. 0.1 N HCl

Found. P 1.45, 1.40; N 1.21

The composition of the phosphatide will be reported in a separate paper.

**Composition of Neutral Fat**—The following constants of the neutral fat were determined by the official methods (5).

Saponification No.....	209.4
Free acid No.....	1.3
I No. (Hanus).....	35.9
Reichert-Meissl No.....	25.2
Polenske No.....	6.1
Unsaponifiable matter, <i>per cent</i> .....	6.7

**Saponification**—The neutral fat, 100 gm., was saponified by refluxing with an excess of alcoholic potassium hydroxide. The cleavage products were separated by the usual methods into 6.76 gm. of unsaponifiable matter, 81.5 gm. of fatty acids, and 12.5 gm. of crude glycerol.

**Unsaponifiable Matter**—The unsaponifiable fraction was a slightly yellowish crystalline mass and it gave a strong positive Liebermann-Burchard reaction, thus indicating the presence of sterols. The substance was dissolved in alcohol and decolorized by treatment with norit. After the solution had been concen-

trated and cooled, a large amount of colorless, thin rectangular plate-shaped crystals separated and after three recrystallizations the snow-white substance weighed 2.5 gm. The crystals melted at 149–150°, solidified at 118°, and remelted at 149–150°.

*Rotation*—0.5961 gm. of substance dissolved in chloroform and made up to 10 cc. gave in a 1 dm. tube an observed rotation of  $-2.35^\circ$  at  $23^\circ$ ;  $[\alpha]_D^{25} = -39.4^\circ$ .

The properties of the crystals together with the melting point and optical rotation show that this substance is cholesterol.

The combined mother liquors from the crystallizations mentioned above yielded on concentration and cooling an additional 0.5 gm. of crystalline cholesterol, m.p. 148–149°. The final mother liquor on evaporation to dryness left a solid wax-like residue. The material gave sterol color reactions but it was impossible to separate any pure crystalline substance from this mixture.

The cholesterol obtained from the unsaponifiable matter was in all probability derived from the skim milk used in the culture medium rather than from the bacteria. Further investigation will be necessary, however, to determine whether the *Lactobacillus* does form cholesterol.

### *Fatty Acids*

The crude fatty acids had a low acetyl value, namely 5.2, but we did not succeed in isolating any hydroxy acid. The acids were separated by means of the lead soap-ether method (6, 7) into 47.1 gm. or 57.7 per cent of solid acids and 30.1 gm. or 36.9 per cent of liquid acids, the latter having an iodine number of 75.6.

*Solid Fatty Acids*—The acids were converted into methyl esters and the esters were carefully fractionated and refractionated at a pressure of 0.005 to 0.001 mm. The purest fractions corresponded to methyl laurate, methyl myristate, methyl palmitate, and methyl stearate. The melting points and indices of refraction were used as criteria of the purity of the ester fractions. The esters were saponified and the free acids were isolated and recrystallized from alcohol. The melting point and mixed melting point of the acids were determined, after which the acids were titrated with 0.1 N alcoholic potassium hydroxide.

*Fraction 1. Methyl Laurate*—The ester melted at  $2-3^\circ$ ,  $n_D^{25}$ ,

1.4290. Methyl laurate melts at 4–5° and  $n_D^{25}$  is 1.4290. The free acid melted at 43–43.5° and showed no depression when mixed with lauric acid. 0.3026 gm. of acid required 15.05 cc. of 0.1 N KOH.

$C_{12}H_{24}O_2$ . Mol. wt. calculated, 200; found, 201

*Fraction 2. Methyl Myristate*—The ester melted at 18°,  $n_D^{55}$ , 1.4241. The free acid melted at 53–54° and showed no depression when mixed with myristic acid, m.p. 54°. 0.3570 gm. of acid required 15.55 cc. of 0.1 N KOH.

$C_{14}H_{28}O_2$ . Mol. wt. calculated, 228; found, 229

*Fraction 3. Methyl Palmitate*—The ester melted at 27–28°,  $n_D^{55}$ , 1.4265. Methyl palmitate melts at 28° and  $n_D^{55}$  is 1.4260. The free acid melted at 62–63° and showed no depression when mixed with palmitic acid. 0.2618 gm. of acid required 10.28 cc. of 0.1 N KOH.

$C_{16}H_{32}O_2$ . Mol. wt. calculated, 256; found, 254

*Fraction 4. Methyl Stearate*—The ester melted at 36–37°,  $n_D^{55}$ , 1.4305. Methyl stearate melts at 38° and  $n_D^{55}$  is 1.4300. The free acid melted at 69–70° and showed no depression when mixed with stearic acid. 0.4012 gm. of acid required 14.05 cc. of 0.1 N KOH.

$C_{18}H_{36}O_2$ . Mol. wt. calculated, 284; found, 285

The crude fatty acids possessed a distinct odor of butyric acid but in the separation of the fatty acids as outlined above no appreciable quantity of butyric acid could be isolated. It is difficult to determine with any accuracy the amount of the different acids that were present because the fractionation is not quantitative. From the amounts of the various ester fractions that were obtained we estimate that the solid fatty acids consisted approximately of lauric acid 5 per cent, myristic acid 10 per cent, palmitic acid 45 per cent, and stearic acid 40 per cent.

*Liquid Fatty Acids*—The acids were reduced with hydrogen and platinum oxide (8) and the reduction product was separated by means of the lead soap-ether method. The solid reduced acids

weighed 26.1 gm., and a liquid acid obtained from the ether-soluble portion of the lead soaps weighed 3.3 gm. The latter had an iodine number of 6 and was optically inactive. The molecular weight determined by titration was 262. The substance was no doubt a mixture, and owing to the small amount available no work was done on this fraction.

*Solid Reduced Acid*—The acid was converted into the methyl ester and the latter was distilled in a high vacuum. All of the ester distilled between 138–143° and apparently was pure methyl stearate. The ester melted at 38–39°,  $n_D^{25}$ , 1.4303. The free acid melted at 69–70° and showed no depression when mixed with stearic acid. 0.3758 gm. of acid required 13.17 cc. of 0.1 N KOH.

$C_{18}H_{36}O_2$ . Mol. wt. calculated, 284; found, 285

The data indicate that the reduced solid acid was stearic acid. From the iodine number of the crude liquid acids and the amount of stearic acid obtained on reduction, it is safe to assume that the unsaturated acid was oleic acid.

#### *Water-Soluble Constituents*

The aqueous solution, after the fatty acids had been extracted with ether, was evaporated to dryness under reduced pressure and the residue was extracted with absolute alcohol in the usual manner. The alcohol was evaporated and the residue was again treated with absolute alcohol; after filtering, the solution was evaporated to dryness. The residual syrup thus obtained weighed 12.5 gm. and gave on heating with potassium bisulfate a strong odor of acrolein. In order to confirm the presence of glycerol, 1 gm. of the syrup was benzoylated by the method of Einhorn and Hollandt (9). The reaction product, after it had been purified by recrystallization from absolute methyl alcohol, weighed 1.2 gm. and melted at 75–76°. There was no depression of the melting point when mixed with glycerol tribenzoate. The results indicate that glycerol is the main constituent of the water-soluble fraction.

We desire to express our thanks to the Mulford Biological Laboratories, Sharp and Dohme, for supplying large quantities of *Lactobacillus acidophilus*.

## SUMMARY

*Lactobacillus acidophilus* contains about 7 per cent of ether-soluble lipids.

The crude lipids are composed approximately of 28 per cent of free fatty acids of which 3.4 per cent is dihydroxystearic acid, 35.2 per cent of neutral glycerides, and 32 per cent of phosphatide.

On saponification the neutral fat gave 6.7 per cent of unsaponifiable matter, 81.5 per cent of fatty acids, and 12.5 per cent of crude glycerol.

The crystalline portion of the unsaponifiable matter was identified as cholesterol.

The fatty acids consisted of 57.8 per cent of solid saturated acids and 36.9 per cent of unsaturated acids.

The saturated fatty acids consisted of lauric, myristic, palmitic, and stearic acids.

The unsaturated acid fraction yielded only stearic acid on catalytic reduction and it is most probable that the unsaturated acid was oleic acid.

A small amount of a liquid saturated fatty acid was present in the unsaturated acid fraction but could not be identified.

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# A CONTRIBUTION TO THE CHEMISTRY OF TOMATO PIGMENTS

## THE COLORING MATTER IN AMERICAN RED AND PURPLE TOMATOES (*LYCOPERSICUM ESCULENTUM*)\*

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The characteristic red pigment of the tomato was first investigated in 1876 by Millardet (1), who obtained it in a crystalline state and also observed the crystals in the flesh of the ripe fruit. He reported that the crystalline pigment, for which he proposed the name solanorubine, was insoluble in water, soluble in hot alcohol, and easily soluble in carbon disulfide, chloroform, and benzene. He also established that absorption in the visible portion of the spectrum of a carbon disulfide solution of the pigment was characterized by two bands in the green region and one in the blue.

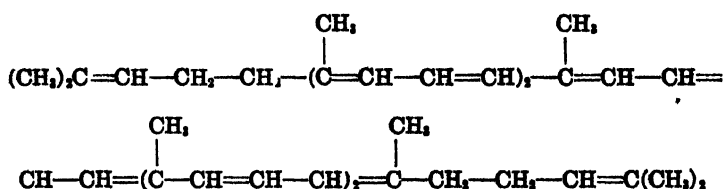
Later investigators (2-5) found carotene present in tomatoes and considered it identical with the pigment obtained by Millardet. Tomato pigment was again studied in 1903 by Schunck (6) who found the red coloring matter clearly distinguishable from carotene in appearance, crystal form, solubility, and absorption spectrum, and named it lycopin. Since the work of Schunck the pigment has usually been referred to as lycopin, although lycopersicin (7) and lycopene have both been used. The latter term is to be preferred since the ending "ene" denotes the unsaturated hydrocarbon nature of the coloring matter.

The first chemical investigation of lycopene was undertaken by Montanari (8) in 1904. His results led him to consider the tomato pigment a condensation product of 2 molecules of carotene, possessing the formula  $C_{52}H_{74}$ .

\* Contribution No. 202 from the Food Research Division.

In 1909 Willstätter and Escher (9, 10) isolated from Italian tomato conserve<sup>1</sup> a relatively large quantity of the pure crystallized red pigment, together with a much smaller quantity of the yellow pigment, carotene. They found that lycopene is an unsaturated hydrocarbon and possesses the same composition and molecular weight as carotene, namely  $C_{40}H_{56}$ . The crystals had a brownish carmine-red color and velvety waxy consistency. Under the microscope they generally appeared as needles or elongated, flat-sided prisms, with split or blunt ends, and often occurred in clusters. Where individual crystals crossed each other, there was observed a deeper red color. Lycopene was less soluble than carotene in ether, carbon disulfide, petroleum ether, and alcohol, and oxidized and bleached more readily.

As a result of the work of Karrer and others (11-16) the following structural formula has been assigned (14) to the pigment.



Willstätter and Escher, as well as Montanari, carried out their investigations on the pigment isolated from Italian-grown varieties. As far as we know, there has been no complete chemical investigation reported on the pigments occurring in tomatoes grown in the United States. It is well known that the best known commercial varieties of Italian tomatoes differ in many respects from domestic varieties. Italian tomatoes are smooth, plum-shaped, and approximately 3 inches long and 1 inch in diameter. As a rule, they have a more brilliant and more uniformly distributed red color than the average tomato grown in the United States. They have firm flesh and practically no core. American-grown tomatoes are large and globular and generally more juicy. While California tomatoes more closely resemble Italian-grown tomatoes in composition, the latter are sweeter and contain less acid than most domestic varieties. In this country two distinct types of red

<sup>1</sup> Società generale delle conserve alimentari cirio, Naples.

tomatoes are recognized. One type is carmine-red with a distinct purplish cast. Tomatoes belonging to this type, which are characterized by purplish red flesh and transparent skin, are sometimes described as purple tomatoes. Typical examples are Livingston Globe and Cooper Special. In the second type the color approaches scarlet-red. These tomatoes are characterized by purplish red flesh and yellow pigmented skin, which together cause the color to appear more nearly true red. The Stone, Indiana Baltimore, and Santa Clara Canner are examples of this type.

Because of the difference mentioned above in appearance of American tomatoes and the more decided differences existing between American- and Italian-grown tomatoes, it was decided to isolate and study the pigment from American red and purple tomatoes and to determine whether it is identical with that isolated from Italian varieties. The results of such an investigation would definitely answer the question often raised by canners as to whether the pigments are the same in American red and purple varieties.

#### EXPERIMENTAL

*Preparation of Crude Pigment*—For the purpose of this study four varieties were used, namely Fiaschetti (Italian-grown red), Indiana Baltimore (American-grown red), Santa Clara Canner (American-grown red), and Cooper Special (American-grown purple). Commercially concentrated tomato pulp, either purée or paste, was used in each case.

The method employed for the isolation of the crude pigment was essentially that of Willstätter and Escher (10). When purée was used the insoluble portion, which contained most of the pigment associated with the plastids, was separated from the largest portion of the juice by centrifuging in a De Laval cream separator, with a clarifying bowl. Further dehydration was effected by the use of 95 per cent alcohol. After the mixture was stirred, the excess aqueous alcoholic portion was removed by means of a hand-press and the process repeated twice. When tomato paste was used, it was necessary to carry out only the alcoholic dehydration process.

The partly dried, dark red material which remained after treatment with alcohol was completely dried in the open on a wire



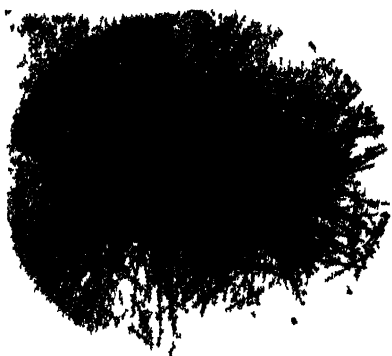


FIG 1



FIG. 2

FIG 1 Rosettes of needles of lycopene from carbon disulfide and petroleum ether ( $\times 28$ )

FIG 2 Elongated, flat-sided prisms of lycopene from carbon disulfide and petroleum ether ( $\times 90$  approximately)

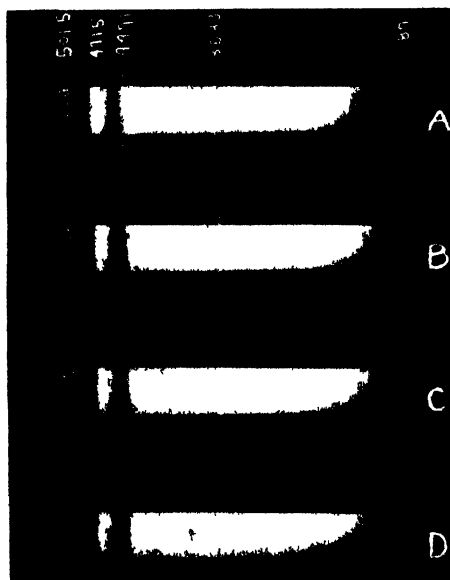


FIG 3 Spectrograms of lycopene from Fiaschetti, *A*, Indiana Baltimore, *B*, Cooper Special, *C*; Santa Clara Canner, *D*. The concentration is 1 mg per 100 cc of an equal mixture of methyl alcohol and ethyl ether; 5 mm thickness; liquid air temperature; 1 minute exposure; tungsten light; helium reference lines

screen at a temperature not exceeding 40°. The product thus obtained was finely ground and extracted with carbon disulfide in a percolator until the extract was practically colorless. The extract was then evaporated *in vacuo* to small bulk at a temperature not exceeding 40°. To this concentrated pigment solution 3 volumes of absolute alcohol were added, and the mixture was kept at low temperature until separation of the semicrystalline pigment occurred. The insoluble pigment was collected by suction on a porcelain funnel under a stream of nitrogen and when thoroughly dried was stored in sealed tubes in the absence of oxygen. In all, 57 gm. of the crude pigment were prepared in this manner.

*Purification for Analysis*—The crude pigment was dissolved in the smallest possible volume of carbon disulfide, and 2 to 3 volumes of redistilled petroleum ether (b.p. 30–60°, known commercially as Skellysolve) were added slowly with shaking. The crystalline product which gradually separated was redissolved in carbon disulfide and reprecipitated with petroleum ether several times. While petroleum ether is superior to alcohol for the crystallization of lycopene from carbon disulfide solution, there is a greater loss due to greater solubility of the pigment in petroleum ether. Care was taken at all stages to protect the pigment from oxidation by keeping both the solution and filtered crystals under an atmosphere of nitrogen. As obtained by this method of purification and crystallization, the crystals of lycopene exhibited a brownish carmine-red color with a metallic sheen and appeared under the microscope as rosettes of needles (Fig. 1) or elongated, flat-sided prisms (Fig. 2).

The pigment from each of the four sources, after being dried in a vacuum desiccator over phosphorus pentoxide, gave the following analysis.

*Fiaschetti*

0.0685 gm.: 0.0651 gm. H<sub>2</sub>O and 0.0224 gm. CO<sub>2</sub>  
0.0676 " : 0.0635 " " " 0.2219 " "  
Found. C 89.18, 89.52; H 10.65, 10.53

*Indiana Baltimore*

0.0680 gm.: 0.0630 gm. H<sub>2</sub>O and 0.2222 gm. CO<sub>2</sub>  
0.0680 " : 0.0648 " " " 0.2232 " "  
0.0764 " : 0.0712 " " " 0.2509 " "  
Found. C 89.12, 89.52, 89.56; H 10.39, 10.68, 10.45

*Santa Clara Canner*

0.0681 gm.: 0.0649 gm.  $H_2O$  and 0.2234 gm.  $CO_2$   
0.0633 " : 0.0589 " " " 0.2073 " "  
Found. C 89.47, 89.31; H 10 68, 10.43

*Cooper Special*

0.0710 gm.: 0.0654 gm.  $H_2O$  and 0.2329 gm.  $CO_2$   
0.0691 " : 0.0642 " " " 0.2275 " "  
Found. C 89.46, 89.79; H 10 33, 10.41  
Found, average of nine determinations. C 89.44; H 10.51  
Lycopene,  $C_{40}H_{56}$ , requires: C 89.48; H 10.52

*Spectral Analysis*

In order to obtain additional evidence of the identity and purity of lycopene from the four sources, samples of each were submitted to spectrographic analysis. The spectrograms shown in Fig. 3 were kindly prepared by G. E. Hilbert and E. F. Jansen of the Bureau of Chemistry and Soils, United States Department of Agriculture, by means of a new low temperature procedure developed by them. An examination of the absorption spectra leaves no doubt that the coloring matters from all four sources are identical.

Fig. 4 represents the visible and ultra-violet absorption spectrum of lycopene which was dissolved in a mixture of ether and alcohol. The data used in plotting this curve were kindly furnished by the United States Bureau of Standards through the courtesy of K. S. Gibson, D. B. Judd, and M. E. Brown. Since lycopene is easily autoxidizable, it was found extremely difficult to prepare solutions entirely free of oxidation. Even the same solution when allowed to remain in the dark overnight showed evidence from the data observed of some oxidation. Changes in the curve due to the effect of slight oxidation were somewhat comparable to those reported by McNicholas (17) for carotene. The visible portion of the curve represents a solution which was considered the best of three samples. Owing to refinements introduced in its preparation and to the nature of the curve obtained, it is believed that very little if any oxidation could have taken place in this solution. While the fullest precautions were exercised to prevent oxidation of the solution used for the ultra-violet measurements, the curve for this portion of the spectrum is probably not quite so accurate as that for the visible end. Furthermore, while observations in

the ultra-violet region indicated an absorption band between the frequencies 1000 and 1050, complete data were lacking. The probable course of the absorption in this region is, therefore, indicated by a dotted line.

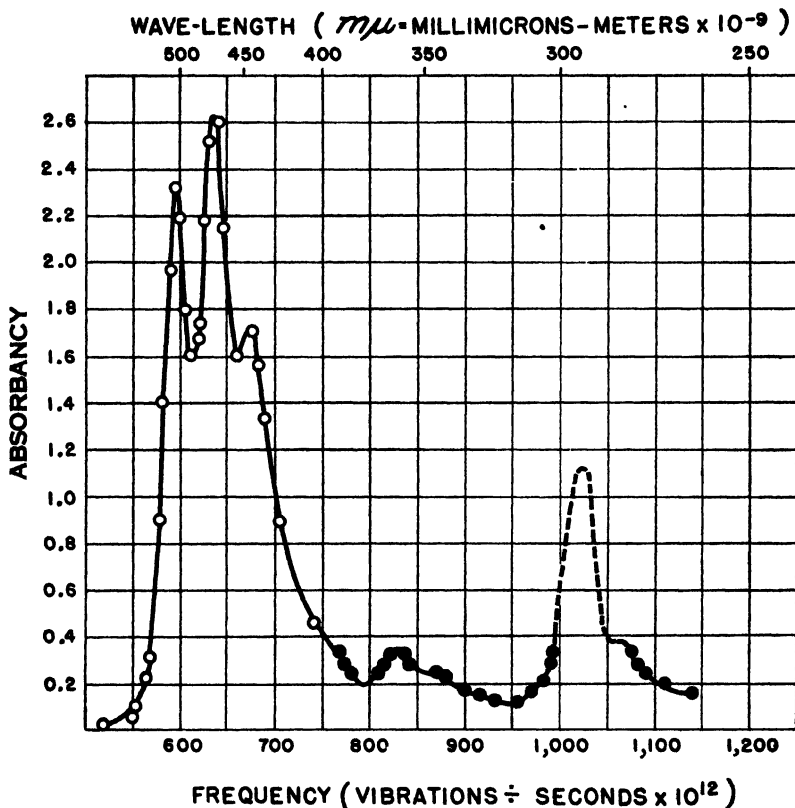


FIG. 4. Visible and ultra-violet absorption spectrum of lycopene. Solvent: 80 per cent alcohol + 20 per cent ether; concentration: 0.40 cg. per liter; thickness: 2 cm.; methods: clear circles, visual; solid circles, photographic. Absorbancy is defined by the relation,  $-\log_{10} (T_{sol.}/T_{sov.})$ , where the transmissions of the cells containing the solution and solvent are represented by  $T_{sol.}$  and  $T_{sov.}$ , respectively; refer to McNicholas (17).

#### SUMMARY

The characteristic red coloring matter in American red and purple tomatoes has been isolated and studied. The pigment

obtained from the American-grown varieties, Indiana Baltimore, Santa Clara Canner, and Cooper Special, was found to be identical with lycopene isolated from an Italian variety known as Fiaschetti.

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## THE IODOMETRIC DETERMINATION OF CYSTINE IN THE URINE

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The iodometric titration of thiol compounds, first introduced by Rosenheim and Davidsohn (1) and modified by Klason and Carlson (2), was applied to the determination of cysteine and related compounds by Okuda, who studied the reaction of cysteine with iodine in acid solution (3). The reaction would be a valuable one if the conditions for the conversion of  $\text{—SH}$  to  $\text{—SS—}$  (cysteine to cystine) could be controlled. In connection with certain metabolic studies concerned with the oxidation of cystine and methionine in the animal organism (4), an attempt was made to use the Okuda method but difficulty was experienced in securing satisfactory values with pure cystine solutions. A detailed investigation of the factors concerned in the more accurate determination of cystine by iodometric titration was accordingly undertaken. As a result of preliminary experiments, the following conditions were found essential: (1) low temperature ( $0^\circ$ ), (2) fairly high concentration of acid (2 per cent hydrochloric acid), (3) presence of amounts of iodine only slightly in excess of those required for the reaction. After satisfactory conditions for the method to be reported in this paper had been worked out, a similar study by Lucas and King (5) appeared in which a study of optimal conditions for accurate iodometric titrations of thiol derivatives was made. Since these workers present an adequate and detailed discussion of the literature, we have omitted further general discussion of iodometry as applied to thiol compounds. Inasmuch as Lucas and King reported the application of their titration to pure solutions only and did not make a study of the conditions as applied to urine, we are presenting our method for the determination of cystine in urine.

*Determination of Cystine (Disulfides) in Urine**Reagents*

1. Iodine in potassium iodide (approximately 0.01 N). 1.27 gm. of pure iodine and 2 gm. of potassium iodide are dissolved in approximately 400 cc. of distilled water. The solution is filtered into a liter volumetric flask and made up to volume. It is not necessary to make this solution exactly 0.01 N because a blank titration with the thiosulfate is made in each determination.

2. Sodium thiosulfate (0.02 N). This solution is prepared and preserved with the usual precautions. Standardization is best accomplished by the use of potassium iodate in the usual way.

3. Starch indicator. This is a 2 per cent solution of soluble starch in a saturated solution of sodium chloride.

4. Hydrochloric acid, approximately 2 per cent.

5. Hydrochloric acid, approximately 10 per cent.

6. Norit.

*Decolorization and Removal of Interfering Substances from Urine*

—To 25 cc. of urine in a 250 cc. beaker are added 15 cc. of 10 per cent hydrochloric acid, 10 cc. of water, and 0.6 gm. of norit. The mixture is boiled for 1 minute, allowed to cool for 10 minutes (decolorization was unsatisfactory if the cooling was omitted), and filtered through a small filter paper in a Buchner funnel. The norit is washed with 10 cc. of 2 per cent hydrochloric acid, and the filtrate and washings are transferred quantitatively with the aid of distilled water to a volumetric flask of 100 cc. capacity, which contains 3 cc. of 10 per cent hydrochloric acid. The contents of the flask are cooled in an ice bath, made up to volume with ice-cold water, and mixed thoroughly. The final concentration of the hydrochloric acid in the solution is approximately 2 per cent.

*Reduction of Cystine*—The contents of the flask are now transferred to a dry cylinder (250 cc. capacity),<sup>1</sup> about 0.5 gm. of zinc dust is added, and the reduction is allowed to proceed for 30 minutes at room temperature. After reduction, the solution is filtered through a dry filter paper with suction into a dry flask, transferred to a dry cylinder, and returned to the ice bath.

While the reduction is proceeding, a series of Erlenmeyer flasks

<sup>1</sup> We have found that the reduction proceeds more satisfactorily in a graduated cylinder than in a volumetric flask.

containing 5 cc. of the standard solution of iodine in potassium iodide is prepared and the contents are frozen in an ice-salt mixture. A blank determination is obtained by adding 15 cc. of 2 per cent hydrochloric acid to one of the flasks containing the standard iodine-iodide solution and titrating with the standard thiosulfate, starch being used as an indicator.

*Preliminary Titration*—As already stated, the titration with iodine is most satisfactorily made when the amount of iodine present in excess of that required for reaction with the sulfhydryl groups is not great. For this reason, a preliminary titration is carried out to determine the approximate amount of iodine required for the reaction. A definite amount of the urine after reduction (we have usually found 15 cc. a convenient volume) is pipetted from the cylinder in the ice bath into a flask containing 5 cc. of the frozen iodine-potassium iodide solution, 0.5 cc. of the starch indicator is added, and the solution is titrated with the standard thiosulfate. The thiosulfate should be added from the burette at such a rate that the blue color developed as the iodine-iodide solution melts is destroyed. The final addition of thiosulfate should be made just as the last of the iodine-iodide solution melts. This titration gives an approximation of the amount of thiosulfate required to react with the iodine not used for the reaction with —SH groups, but will be slightly less than the amount needed in the final more exact titration, since in the presence of an excess of iodine, the reaction with the sulfhydryl tends to proceed beyond the formation of the disulfide and slightly more iodine will be required.

*Final (Accurate) Titration*—From the burette an amount of thiosulfate slightly less (e.g., 0.05 cc. less) than that used in the preliminary titration is run into another flask containing the frozen iodine-iodide solution, starch is added, and an aliquot of the ice-cold solution (usually 15 cc.) to be analyzed is pipetted into the flask which is shaken until the ice is melted. As fast as the blue color appears, thiosulfate is added to react with the free iodine. The titration should be completed as the last of the frozen iodine-iodide solution melts. With an accurate microburette,<sup>2</sup> duplicate determinations agreeing within 0.02 cc. can usually be obtained.

<sup>2</sup> We have found the Koch microburette (Arthur H. Thomas Company) graduated in 0.01 cc. satisfactory.



*Calculation*—1 cc. of 0.02 N iodine solution is equivalent to 2.40 mg. of cystine or 2.42 mg. of cysteine. If 15 cc. aliquots of the solution of the reduced urine after charcoal treatment are used, the calculation is as follows:  $(a - b) \times 100/15 \times 2.40$ , where  $a$  is the blank titration of the iodine-iodide solution in terms of 0.02 N thiosulfate and  $b$  is the amount of thiosulfate required to titrate the excess of iodine in the unknown. The value obtained represents the mg. of cystine in 25 cc. of the urine.

#### DISCUSSION

The accuracy of the method was checked with solutions of pure cystine in dilute hydrochloric acid, treated as described except that the preliminary treatment with norit was omitted. The theoretical amount of 0.02 N iodine required to titrate the thiol groups resulting from the reduction of 2 mg. of cystine was 0.833 cc. The values actually observed over a range of concentration of from 0.8 to 4.0 mg. of cystine were from 0.805 to 0.850 cc. per 2 mg. of cystine, with an average of 0.835 cc. The use of the theoretical equation for the calculation of results seemed justifiable and the use of a titration curve as recommended by Okuda was considered unnecessary.

We were concerned primarily with the analysis of urine from rabbits of about 2 to 3 kilos in weight, fed a diet of oats and cabbage. After the dilution of these urines to 150 cc., the 25 cc. aliquots mentioned were used for the analysis. If the volume of the urine was greater than 150 cc., the amount of norit used for the decolorization of the 25 cc. aliquot was diminished proportionally.

The optimal quantities of norit and acid to be used in the decolorization of the urine were determined by experiments with a considerable number of urines, both human and rabbit. With human urines diluted to 1500 cc., it was found that decolorization of a 25 cc. sample could be effected satisfactorily by the procedure outlined. No satisfactory recoveries of cystine could be obtained with dog urine. Other decolorizing carbons could undoubtedly be used, but optimal conditions must be determined for them.

In a series of experiments with 20 mg. of pure cystine, the per cents of cystine recovered after treatment with 0.5, 1.0, 1.5, and 2.0 gm. of norit were 87, 66, 57, and 48, respectively. In urine

acidified as described, norit in the amount recommended appears to remove pigments and other interfering substances without loss of cystine. With larger amounts of norit, losses of cystine added to urine were observed.

In urine, reducing substances are normally present which react with considerable amounts of iodine. Treatment of the acidified urine with norit in the amount recommended removed these substances, so that no iodine was used in the titration of the filtrates obtained if reduction by zinc was omitted. These results were so consistent that titrations for cystine (*i. e.*, without reduction by zinc and hydrochloric acid) have been omitted except in certain feeding experiments where the presence of cystine or a derivative was anticipated. The nature of these reducing substances is not known. The failure of normal urine to give a positive ammonium hydroxide-nitroprusside test indicates that mercapto derivatives are not concerned to any important degree.

In Table I are presented the results of a series of experiments in which the recovery of cystine added to urine was studied. The results show satisfactory recovery of cystine and require little comment. When the method was applied to normal human urine, values indicating a normal content of cystine of from 1.1 to 7.5 mg. of cystine per 100 cc. of urine were obtained. In two urines of supposedly normal individuals, higher values, 11.8 and 12.5 mg., were found. We have also determined the cystine content of cystinuric urines by this method. The cystinuric urines examined ranged from those which gave weakly positive cyanide-nitroprusside tests to definitely cystinuric urines, in which the diagnosis of cystinuria was made by microscopic identification of cystine crystals in the urine. Values ranging from 10.2 to 96.5 mg. of cystine per 100 cc. of urine were obtained.

It is realized that satisfactory recovery alone does not constitute an accurate criterion of a method. However, in view of the known relatively low concentration of cystine in normal urines, we believe that the results obtained by this method afford a more satisfactory indication of the cystine (and disulfide) content of urine than the results obtained by the application to the urine of the other common methods. It is true that the method is not entirely specific for cystine. The same objection, however, applies to the other common methods for cystine determination except that of Sullivan,

the practical application of which to urine offers difficulties. The method described has the advantages over the Okuda procedure for urine in a more clear cut end-point, more adequate control of temperature and other conditions which makes possible a stoichiometric reaction and renders unnecessary the use of a correction

TABLE I

*Recovery of Cystine Added to Normal Rabbit Urine and to Normal or Cystinuric Human Urine*

All results are averages of duplicate titrations. Aliquots of 20 cc. were titrated. Although 0.01 N iodine solution was used, since the back titrations were made with 0.02 N thiosulfate, the iodine used is calculated as 0.02 N. The first group of urines includes normal rabbit urines; the second group includes human urines, both normal and cystinuric.

Added cystine	Standard thiosulfate (0.02 N) required for back titration of urine		Iodine (0.02 N) used by added cystine	Cystine recovered	
	Before cystine	After cystine			
mg.	cc.	cc.	cc.	mg.	per cent
4	2 36	2 03	0 33	4.00	100
4	2 58	2 24	0 34	4 08	102
4	2 62	2 29	0 33	4 00	100
12	2 36	1 33	1 03	12 36	103
20	2 36	0 66	1 70	20 40	102
20	2 36	0 76	1 60	19 20	96
20	2 62	0 92	1 70	20 40	102
20	2 58	0 83	1 75	21 00	105
4	2 62	2 29	0 33	3.96	99
4	2 67	2 34	0 33	3.96	99
20	2 62	0 90	1 72	20.64	103
20	2 67	0 98	1 69	20.28	101
4*	0 49	0 16	0 33	3.96	99
4*	1 86	1 52	0 34	4.08	102

\* Cystinuric urine.

curve, and in satisfactory removal of interfering substances without loss of cystine.

Since we wished to apply this method to the determination of cystine or related compounds containing the disulfide linkage in urine after feeding cystine and methionine to rabbits (4), it was

necessary to determine the reaction of methionine and its demethylated oxidation product, homocystine (6), in the method outlined. Methionine gave no titration value either before or after reduction. Homocystine contains a disulfide linkage; it should therefore be quantitatively determined by the proposed method. We prepared homocystine from methionine according to the procedure of Butz and du Vigneaud (6) and obtained a white crystalline material containing 23.4 per cent of sulfur (theory, 23.8 per cent), which gave a positive reaction with cyanide and nitroprusside and with the Folin-Marenzi reagent (7), but a negative Sullivan test. 18.7 mg. of this product were used for the titration, which indicated the presence of 19.0 mg., a recovery of 101 per cent. We have been particularly interested in the values obtained with methionine and homocystine, since, as reported elsewhere (4), after the administration of methionine to rabbits, the urines contained a substance which reacted qualitatively as did homocystine, and in these urines increased iodometric values by the method outlined were also observed.

#### SUMMARY

A modification of the Okuda iodometric titration method for the determination of cystine (and disulfides) in urine is presented. The optimal conditions for the reaction between —SH groups and iodine have been found to be (1) low temperature (0°), (2) acid reaction (2 per cent hydrochloric acid), and (3) avoidance of amounts of iodine in any considerable excess of those required for the reaction. This method has been used successfully with rabbit and human urine (normal and cystinuric).

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## VITAMIN E

### I. SOME CHEMICAL AND PHYSIOLOGICAL PROPERTIES

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The only published systematic investigation of vitamin E is that of Evans and Burr (6). Evans (5) has recently reviewed the literature on the subject. The data herein recorded represent investigations during the course of 4 years on some of its physical, chemical, and physiological properties. The most striking function of vitamin E is to provide for a normal gestation in a pregnant rat, in that it prevents the resorption of the embryos which invariably occurs in its absence. A curative method of assay based upon this physiological property was developed by Evans and Burr (6) and has been used throughout the experiments to be described.

*Assay*—Female rats weighing 30 to 40 gm. were placed on a diet composed of sucrose 46, lard 22, casein (commercial) 18, dried yeast<sup>1</sup> 8, salts<sup>2</sup> 4, and cod liver oil 2 parts, *ad libitum*. At 70 to 90 days of age (150 gm.), daily weighings and vaginal smears were begun, and at the next estrous period, the rats were mated with normal, healthy males. The first gestation was a typical resorption characteristic of a deficiency in vitamin E. There has been only one case of "initial fertility" in more than 200 female rats whose histories have been followed. When the estrous cycle returned, the animals were again mated. If the mating was positive as indicated by the presence of sperm in the smear, the food was removed from the cage, and 10 to 12 hours later, the material to be assayed for its content of vitamin E was mixed with a small amount of the basal diet and offered to the animal. Small doses

<sup>1</sup> Kindly furnished by the Northwestern Yeast Company.

<sup>2</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **32**, 317 (1917). More recently, Hawk, P. B., and Oser, B. L., *Science*, **74**, 369 (1931) with increase from 4 to 4½ parts.

were eaten immediately. If the animal gave birth to young, they were reduced to six, and the mother was allowed to suckle them as long as she would or until they could be weaned. The animal then underwent another resorption before it was used again for an assay. If the feeding resulted in a resorption, it could be utilized at once for another test. At times positive matings occurred although no spermatozoa were detected. Such a condition was recognized by the failure of the cycle. If the animal was available for a test, having previously undergone a resorption, the test material was administered on the 5th day after the end of the previous period. It is fortunate that the estrous cycle of the rat is slightly shorter than the limiting period ((6) p. 106) within which time test substances may be fed and still be effective. A pregnant condition was always verified by the finding of the vaginal blood leak on the 12th to 14th day after impregnation. Each animal was used during the period of six to eight gestations. More vitamin E seems to be necessary for successful gestations in older animals (unpublished observations).

*Relation of Amount of Vitamin E Administered to Duration of Fertility and to Survival of Young*—Numerous isolated observations and one rather complete series of experiments confirm Evans and Burr's observations: (1) that the administration of large amounts of vitamin E to animals on a vitamin E-deficient diet does not improve the reproductive mechanism beyond normal limits, as judged by the number of young ((6) p. 94); (2) that the administration of several times the minimal effective dose of vitamin E on the 1st day of a trial gestation can be expected to result in the preservation of fertility for a second gestation, but not for a third ((6) p. 84); (3) that the addition of other factors besides vitamin E is necessary to insure lactation (7).

Table I indicates the results obtained on a series of ten animals given graded doses of a crude fraction, Concentrate W2-4, from wheat germ oil. A minimum of 20 mg. was necessary for a successful gestation. If 30 mg. or more were administered, fertility was preserved for a second gestation except in the case of Rat 450.  $5\frac{1}{2}$  times the minimal effective dose was not sufficient to carry fertility over to a third gestation. There is apparently no correlation between the amount of vitamin administered and the number of young or their survival. A few young were weaned from a

second gestation in which the amount of vitamin E present in the mother must have been less than when the first litter was cast, of which none was weaned. The factor necessary for lactation is probably an increase in vitamin B (7). Apparently even 8 per cent dried yeast in the diet is insufficient. When additional yeast is fed separately, lactation is normal.

*Observations on Paralysis in Suckling Young of Rats on Vitamin E-Deficient Diet*—During the course of these investigations, oppor-

TABLE I

*Number of Young Born to Female Rats on Vitamin E-Deficient Diet after Administration of Single Doses of Concentrate W2-4*

Rat No.	Dose administered	No. of rats in litter			Remarks
		1st gestation	2nd gestation	3rd gestation	
	mg				
440	0	0			
477	10	0			
432	20	6	0		
430	30	7	4	0	
436	40	5	6	0	
438	50	11	13	0	3 weaned of 2nd litter
450	60	3	0		
428	70	6	9	0	
448	80	9	7*		2 " " 1st "
431	90	11	8	0	2 " " 2nd "
439	110	8	4	0	

\* Uterine hemorrhage at parturition. Fatalities from this cause are not frequent but occur regularly among female rats on a vitamin E-deficient diet. This condition was first noted by Nelson, Jones, Heller, Parks, and Fulmer (13).

tunity was afforded to observe the paralysis in thirty to forty young of mothers fed a vitamin E-deficient diet. The observations confirmed in every way those made by Evans and Burr (8). Attempts to alleviate the symptoms by the administration, orally or subcutaneously, of very active concentrates of vitamin E were ineffective. Animals recovered or succumbed irrespective of the treatment afforded. The paralysis usually appears about the 4th week, and Morelle (12) has recently suggested that the administration of vitamin E must be initiated before the end of the 2nd week to be effective.



*Properties of Vitamin E Concentrates from Lettuce*<sup>3</sup>—A detailed study of the chemical properties of vitamin E was first begun on material obtained from lettuce.<sup>4</sup>

The unsaponifiable lipids of lettuce were prepared and fractionated by the method already described (14). When no further separation could be effected by fractional crystallization from

TABLE II  
*Vitamin E Content of Various Fractions after Vacuum Distillation of a Concentrate from Lettuce*

Distillation A Fraction No.	Temperature range (0.1 mm.)	Amount fed	No. of young	Distillation B Fraction No.	Temperature range (0.1 mm.)	Amount fed	No. of young
	°C.	mg.			°C.	mg.	
4	190–211	10	0	3	182–202	15	10
		15	3			50	6
		25	13	4	202–218	15	4
5	211–225	15	7			25	6
		40	8	5	218–240	25	0
6	225–250	25	0			50	0
		50	1				

various solvents, the remaining highly colored oil was distilled *in vacuo*. The vitamin E content of the various fractions is indicated in Table II. Fractions boiling below 180° (0.1 mm.) were inactive.

<sup>3</sup> A report of this work was presented before the American Society of Biological Chemists at Philadelphia, April 28, 1932 (Olcott, H. S., *J. Biol. Chem.*, **97**, x (1932)).

<sup>4</sup> Since that time and in connection with further work reported in this paper, several oils and unsaponifiable concentrates have been assayed. The results may be briefly summarized as follows: Palm oil contains little or no vitamin E, 10 gm. being insufficient to produce a litter. Palm kernel oil is likewise deficient. A single administration of 3.5 gm. of crude cottonseed oil gave a litter of seven. The crude unsaponifiable lipids of cottonseed oil, after most of the sterols had been removed, gave litters of nine each after the administration of 25 and 50 mg.; 15 mg. were not sufficient for a positive gestation. A fraction obtained in the same way from a hydrogenated cottonseed oil was similarly active, 50 mg. resulted in a litter of seven. It appears that cottonseed oil is richer in vitamin E than has heretofore been supposed. The unsaponifiable lipid fraction of olive oil, after removal of the sterols, contained sufficient vitamin E so that 100 mg. allowed the birth of a litter of six; 50 mg. were without effect.

The results show that the vitamin was not isolated in any one fraction of 20° range. The active portion distilled between 190–220° at approximately 0.1 mm. The temperature range is somewhat, but not significantly, lower than that recorded by Evans and Burr ((6) p. 143) who found that the active fraction from wheat germ oil distilled at 200–233° (0.5 mm.), and that on refractionation, only the distillate from 225–230° (0.01 mm.) retained its activity.

The active distillates were further freed of extraneous material by crystallizations from small amounts of acetone. Additional amounts of sterols and other alcohols of high molecular weight were removed. In this way concentrates were prepared which were active in doses of 10 mg.; in one case 5 mg. allowed the birth

TABLE III  
*Vitamin E Content of Lettuce Concentrates*

	Amount fed	No. of young		Amount fed	No. of young
	<i>mg.</i>			<i>mg.</i>	
Fraction A-4	10	5	Fraction B-4	5	0
“ A-5	3	0		5	0
	3	0		5	0
	5	0		10	7
	5	2		10	5
	10	7			

of a litter (Table III). The minimum effective dose of Evans and Burr's ((6) p. 141) most active material was also between 5 and 10 mg. Evans (5) has indicated that a more active concentrate, with a minimum dose of 1 mg., has recently been obtained by Cornish.

The concentrates from lettuce were light brown oils, viscid in the cold, fluid when warm, which deposited no crystals even on long standing. They were soluble in all organic solvents. Some data on the physical and chemical properties are given in Table IV, along with similar data for concentrates from wheat germ oil for comparison. The striking differences in iodine numbers are probably not real. There is always difficulty in obtaining consistent iodine numbers of unsaponifiable lipid fractions by any method. The data strongly suggest that the vitamin obtained from lettuce is similar to that obtained from wheat germ oil.

The ultra-violet absorption of the lettuce concentrate was studied by means of a Hilger E-3 quartz spectrograph in conjunction with a sector photometer.<sup>5</sup> There was a definite absorption increasing with decreasing wave-length but no bands were discovered. This is in harmony with the observations of Evans and Burr ((6) p. 144). It is unlikely that the absorption bands examined by Bowden and Moore (1) in wheat germ oil concentrates (not assayed) are connected with the biological activity.

TABLE IV

*Comparison of Physical and Chemical Properties of Vitamin E Concentrates from Lettuce and Wheat Germ Oil*

	Source		
	Lettuce	Wheat germ oil	Wheat germ oil (Evans and Burr (6))
C, per cent. . . . .	82 5*	82 5†	81 7
H, " " . . . . .	12 1*	11 4†	12.2
Molecular weight. . . . .		380†	400
Refractive index (20°) . . . . .	1 5060	1.5254	1 5009
Boiling point, °C. . . . .	190-220 (0.1 mm.)	195-220 (0.1 mm.)	200-233 (0.5 mm.)
I No. . . . .	90†	130§	220†
Minimum dosage, mg. . . . .	5-10	3-5	5-10

\* Research Service Laboratories.

† Dr. Ing. A. Schoeller.

‡ Hübl's method.

§ Method of Ralls, J. O., *J. Am. Chem. Soc.*, **55**, 2083 (1933).

*Properties of Vitamin E Concentrates from Wheat Germ Oil*—Inconveniently large quantities of lettuce were required to yield any adequate amount of vitamin E for study. We therefore turned to wheat germ oil which Evans and Burr ((6) p. 67) found to be the most satisfactory source. The procedure outlined by these investigators has been improved by the early use of vacuum distillation in the process. More potent concentrates have been obtained with fewer operations.

Wheat germ oil is saponified with alcoholic potassium hydroxide. The saponification mixture is diluted and thoroughly extracted with ether. The ether extracts are combined, dried, and the ether

<sup>5</sup> With the assistance of Mr. D. C. McCann.

removed by distillation from a steam bath. The unsaponifiable lipids are dissolved in petroleum ether and the sterols which separate are removed by filtration. The petroleum ether is removed from the filtrate; the residue is then extracted with hot methanol. The soluble fraction is poured off the insoluble oil which is twice again extracted with hot methanol. The methanol fractions are cooled and filtered from the sterols which separate. The methanol is removed from the filtrate *in vacuo*. The procedure up to this point is the same as that recommended by Evans and Burr. Without further treatment the residue is then distilled *in vacuo*. The fraction which distills from 200–225° at 0.05 to 0.1 mm. contains the vitamin. These distillates have been uniformly active in amounts as small as 10 mg.

It has been possible further to concentrate the vitamin from this fraction by removing the insoluble material which separates out of an acetone solution at  $-80^{\circ}$ . The results of the assay of one fraction (Concentrate W4-12-2) so purified are shown in the following tabulation.

Amount fed	No. in litter
<i>mg</i>	
5	5
5	10
3	2
3	0
2	0

Further concentration is possible by the adsorption of impurities on alumina, and experiments along this line are in progress.

For purposes of comparison, some of the physical and chemical properties of this material were determined and are given in Table IV.

*Some Chemical Properties of Vitamin E<sup>a</sup>*—The availability of relatively large amounts of vitamin E concentrates has made possi-

<sup>a</sup> Some preliminary experiments were made while the junior author was holder of a National Research Council Fellowship in Medicine at Yale University, 1931–32, in the Department of Physiological Chemistry. Dr. R. J. Anderson of the Department of Chemistry provided some of the facilities of his laboratory. The animal assays in this connection were made in Iowa City.

ble a more extensive study of some of the chemical properties of the vitamin. Evans and Burr's ((6) p. 128) observations regarding the stability of vitamin E to heat and light, and in the ordinary laboratory manipulations, have been confirmed. Bromination destroys the vitamin.

In contrast to bromination, hydrogenation of the concentrates like hydrogenation of the original oils has no effect on the activity. However, the hydrogenation of the concentrates is incomplete. The most active fractions resist the addition of any hydrogen; after long continued shaking with a platinum catalyst in the presence of the gas, no decrease in iodine number could be detected. A concentrate was subjected to 125 atmospheres of hydrogen in the presence of a Raney nickel catalyst at 50° without hydrogenation<sup>7</sup> or inactivation. That vitamin E is resistant to hydrogenation has also been recently suggested by Bowden and Moore (1).

It has not been possible to confirm the single observation of Evans and Burr ((6) p. 123) on the destructive effect of acetylation. Exhaustive acetylation with boiling acetic anhydride, acetyl chloride, or by long standing with pyridine-acetic anhydride mixtures has been ineffective in destroying the activity. A measure of the degree of acetylation is afforded by the presence of an inhibitor in the concentrate, the activity of which can be measured (10). Acetylation destroys completely the effectiveness of the inhibitor. Table V illustrates the activity of an acetylated concentrate. Benzoylation with benzoyl chloride in pyridine likewise did not destroy the vitamin.

Vitamin E is similarly resistant to a mild oxidizing agent. When a concentrate was heated with a methanol solution of silver nitrate, some reduction occurred. The material was evaporated to dryness and taken up in petroleum ether. The solvent was washed with water and evaporated. The recovered material was still active.

A more powerful oxidant destroys the vitamin. Potassium permanganate in pyridine solution was rapidly reduced in the cold by an active concentrate. An excess of the reagent was added and allowed to stand overnight. The  $MnO_2$  was filtered off, the pyri-

<sup>7</sup> We are indebted to Dr. Homer Adkins of the University of Wisconsin.

dine solution evaporated to dryness, and the residue dissolved in petroleum ether, washed with water, and evaporated. The recovered material was inactive.

*Susceptibility of Vitamin E to Oxidative Destruction in Rancid Fats*—The oxidative destruction of vitamin E in the presence of rancid fats has been the subject of several publications which have been reviewed by Cummings and Mattill (2) and Evans (5). The destruction is so marked that Mattill and coworkers were able to render vitamin E-deficient a diet originally adequate merely by the addition of more lard to it (11), and to correlate the vitamin E content of several diets with their susceptibility toward oxidation (9). Evans and Burr (5) also found that vitamin E was sensitive to even slight rancidity changes in fats, although concentrates appeared to be able to withstand drastic oxidation, for example by a stream of air at 97° for 12 hours without destruction. Waddell and Steenbock (16) completely destroyed vitamin E by evaporating an ether solution of  $\text{FeCl}_3$  on an otherwise adequate diet. The evidence at that time suggested that the stability of vitamin E is dependent upon the presence or absence of natural inhibitors of oxidation which are closely associated with it in nature (2), and that studies on the stability of the vitamin must take into account these accompanying factors.

The experiments to be described were designed to confirm the destruction of vitamin E in rancid food mixtures. The results indicated a surprising *resistance* to oxidation. The concentrate used, No. W3-18, prepared from wheat germ oil, was active in a single dose of 8 to 10 mg., and contained an inhibitor for fat oxidation. Previous work had shown that the antioxidant activity could be completely destroyed by acetylation while the vitamin was not affected. Two diets were then prepared, one containing an acetylated portion, and the other an untreated portion of Concentrate W3-18. The diets were exposed to the air at room temperature in open beakers. The food mixture containing the antioxidant remained sweet throughout the duration of the experiment, while that without protection became distinctly rancid on the 11th day.

The animals in the first series were given amounts of the experimental diets corresponding to 30 mg. of the concentrate on the day of positive mating. Since all the gestations were complete,

a second set of diets was prepared and assayed. In this case the animals were given the equivalent of only 10 mg. of the original concentrates. The data, as summarized in Table V, show that even in the absence of an inhibitor, purified concentrates of vitamin E appear to retain their activity for as long as 4 weeks at room temperature in a rancid fat mixture. We are unable at present to reconcile these results with those of previous researches.

*Effect of Vitamin E on Ovaries, and on Cornification and Opening of Vaginal Orifice in Immature Rats*—Several investigators, especially Verzar and coworkers (15), have suggested a relationship

TABLE V  
*Permanence of Vitamin E in Rancid Food Mixtures*

Amount of concentrate fed	Time after preparation of diet	No. of rats in litter	
		Diet A	Diet B
mg.	days		
30	13		7
30	14	5	
30	15	6	
30	20		9
30	26	4	
10	12	1	
10	23	2	5
10	24	2	
10	38	4	

Diet A contained the acetylated concentrate and was rancid on the 11th day. Diet B remained sweet throughout the experiment.

between vitamin E and the sex hormone of the anterior pituitary. Verzar claimed that wheat germ oil concentrates containing vitamin E, like the sex hormone, caused a hypertrophy of the uterus in infantile rats. The hair of males fed the vitamin E-deficient diet became soft, which was also the case with hypophysectomized rats. He drew the conclusion that a lack of vitamin E disturbs the production of the hormone. Since concentrates of vitamin E<sup>8</sup> and a tested preparation of hebin<sup>9</sup> were available, a few parallel obser-

<sup>8</sup> Concentrate W3-18, minimum dose, 8 to 10 mg.

<sup>9</sup> Prepared from urine of pregnant women. We are indebted to Mr. C. A. Pfeiffer and Dr. A. C. Kuyper for the preparation used. The aqueous solution was crystal-clear and contained 100 units per ml.

vations were made on the effect of injections of these on immature rats.

Six female rats, 18 days old, weighing 25 to 30 gm., were used. The vitamin E preparation was dissolved in a small quantity of olive oil, and an amount equivalent to 100 mg. was injected subcutaneously into each of three; two others were each given 20 units of hebin in the same manner; the remaining animal was not treated. At the end of 4 days, the vaginas of the two hebin-treated animals and that of the control animal were open. The animals were killed and the uterus and ovaries were examined.

TABLE VI  
*Effect of Vitamin E and Hebin on Opening of Vagina in Immature Rats*

Initial weight	Material injected	Vaginal opening	Type of smear
<i>gm.</i>		<i>days</i>	
30	Hebin	4	Estrus
29	"	4	"
32	"	3	"
32	"	3	"
34	Vitamin E	11	Anestrus
31	" "	8	"
30	" "	7	"
31	None	8	"
29	"	9	"
34	"	8	"

The uteri of the two animals given hebin were hypertrophied while those of the other four were normal. One ovary of the control, one of a rat given vitamin E, and one of a hebin-treated animal were sectioned and examined.<sup>10</sup> Only the ovary of the rat given hebin showed maturing follicles.

Since the vaginal orifice of the control was open at the end of 4 days, a further series of ten females, all 20 days old, was treated similarly and the time of opening of the vagina determined. The results are summarized in Table VI.

Coupled with the knowledge that a lack of vitamin E in no way disturbs the ordinary sex cycle in the rat ((6) p. 2) such experiments indicate that vitamin E and the sex hormone of the pituitary play entirely different rôles in the physiology of reproduction in

<sup>10</sup> Mr. C. A. Pfeiffer examined the ovaries.



the female. Recently Diakov and Krizenecky (3) have also reached this same conclusion. The wheat germ oil which they used as a source of vitamin E contained only small amounts of the vitamin. It should perhaps be emphasized that all samples of wheat germ oil are not equally rich in vitamin E; some may lack it entirely ((6) p. 102). Experiments on vitamin E without adequate biological assays are valueless.

*Alleged Relationship between Xanthophyll and Vitamin E*—Von Euler and Klussman (4) suggested a relationship between xanthophyll and vitamin E based upon: the similar distribution of the two in nature; the discovery of absorption bands characteristic of xanthophyll in the absorption spectrum of crude vitamin E concentrates; the ease of oxidative destruction of both when the oxidation is catalyzed by small amounts of iron or unsaturated fatty acids. That xanthophyll is not present in the most active vitamin E concentrates is indicated by their lack of color and by the fact that no bands characteristic of xanthophyll are recognizable in the absorption spectra of such concentrates. Each of two female rats on the vitamin E-deficient diet was fed 10 mg. of xanthophyll on the day of a positive mating. Typical resorptions resulted. There is apparently no immediate relationship between xanthophyll and vitamin E. Carotene, fed in 5 mg. doses, was also ineffective.

#### SUMMARY

Various aspects of the chemical and physiological problems concerned with vitamin E have been examined. A curative method of assay was used. A single large dose of vitamin E allows fertility for two gestations, but not for three. Mothers on a vitamin E-deficient diet require more than 8 per cent of yeast in the diet for normal lactation. The paralysis in the young cannot be cured by the administration of vitamin E after the symptoms have appeared; spontaneous recoveries have been observed. An active concentrate was prepared from lettuce by methods of fractional crystallization and distillation. A shortened method is described for preparing concentrates from wheat germ oil by similar procedures. Some of the properties of these concentrates are tabulated. Vitamin E is destroyed by bromination, but not by acetylation, benzylation, mild oxidation with  $\text{AgNO}_3$ ,

or hydrogenation. The concentrates strongly resist saturation with hydrogen. Potassium permanganate destroys the vitamin. Concentrates of vitamin E are stable for as long as 4 weeks in a rancid food mixture. When injected subcutaneously, vitamin E has no effect on the ovaries, uterus, opening of the vagina, or cornification in the immature rat. There is no immediate relationship between xanthophyll and vitamin E.

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## CAROTENE

### VII. PHYSICAL PROPERTIES OF CAROTENES FROM DIFFERENT PLANT SOURCES

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Recently it has been shown that carotene derived from different leaves and from carrot roots possesses the same degree of unsaturation (1-3). On the basis of optical activity, however, it is clearly evident that at least two isomeric carotenes exist, namely  $\alpha$ -carotene which is optically active and  $\beta$ -carotene which is optically inactive (4). Carotene isolated from plant sources often is a mixture of these two forms. In order to define the composition of such carotene samples in terms of their components it is necessary to know the physical properties of the mixtures as well as of the pure components. For this reason several of the physical properties of carotene samples, varying in composition from pure  $\alpha$  to pure  $\beta$ , have been measured and compared with their optical activities. These comparisons have aided in determining the purity of carotene samples as well as in defining their composition.

#### *Optical Rotations*

The  $\alpha$ -carotene used was prepared by the method of Kuhn and Brockmann (5). 3.07 gm. of carotene,  $[\alpha]_{678} = +123^\circ$ , from carrot roots<sup>1</sup> were divided into three portions. Each portion was dissolved in 800 cc. of petroleum ether (b.p. 45-70°) and treated with 10 to 15 gm. of fullers' earth (floridin xxF). The unadsorbed carotene was recovered and recrystallized from *n*-heptane by dissolving in methylene chloride, adding *n*-heptane, and evaporating the methylene chloride. (Total yield 0.424 gm., m.p. 158.5°,

<sup>1</sup> Recovered from mother liquors of carrot root carotene.

$[\alpha]_{5878} = +213^\circ$ .) Two further adsorptions, followed by two final recrystallizations from *n*-heptane, yielded 0.028 gm. of carotene, with m.p. 181–182.5° and the following specific rotations.

*Specific Rotations in Benzene*

Wave-length	Weight of sample	Observed $\alpha$	$[\alpha]$	Temperature
Å.	gm.	degrees	degrees	°C.
7065	0.0275	+0.545	+297	19.0
6678	0.0275	+0.646	+352	18.5
5876	0.00916	+0.357	+584	18.8

Recovered from the optical rotations and recrystallized from *n*-heptane in the manner described,  $\alpha$ -carotene melted at 182.3–182.7° and showed the following composition.

$C_{40}H_{56}$ .	Calculated.	C 89.48, H 10.52
	Found.	" 89.47, " 10.51
		" 89.51, " 10.65

The weighted mean results for the specific rotations of a second sample of  $\alpha$ -carotene prepared in a similar manner were found to be:

Wave-length, Å.....	7065	6678	5876
Specific rotation (16–19°), degrees.....	+311	+354	+593

These results are very nearly the same as the results of Karrer and Walker (6) at shorter wave-lengths but show considerable deviation in the red end of the spectrum.

*Leaf Carotenes ( $\beta$ -Carotene)*—Since the nature of the carotenes from only a few leaf sources had been examined, crystalline carotenes were isolated from several leaves (1) and their physical properties examined. These carotenes are all high melting and optically inactive, indicating that they are  $\beta$ -carotenes.

The summary of results on rotatory power is given in Table I. If any optically active carotene had been present in the leaf extracts it would have concentrated in the mother liquors during the preparation. Therefore, the carotene was recovered from the mother liquors from sunflower leaves. It too was found to be optically inactive (Table I, Experiment 9).

TABLE I

*Optical Rotations of Some Leaf Carotenes in Benzene*

The specific rotation in each experiment was 0°.

Experiment No.	Source of sample	Weight	Angle observed	Melting point (corrected)
		gm.	degrees	°C.
1	Sugar beet leaves	0.0652	-0.001	(182.0)
2	Spinach	0.0467	-0.002	179.6
3*	"	0.0190	-0.007	(179.1-180.1)
4*	Alfalfa	0.0257	+0.005	(179.7-180.2)
5*	Chard	0.0372	-0.001	(180.2-180.8)
6	"	0.0478	+0.006	182.0-183.0
7*	Cauliflower	0.0464	-0.002	181.3-182.5
8	Sunflower	0.0531	+0.005	178.5
9	"	0.0348	+0.003	179.0-180.0

\* These carotene samples were taken from the same lots as were used in the hydrogenation experiments already reported (1). The melting point figures enclosed in parentheses were the original melting points of the carotenes and were not taken just before these measurements. Lycopene was also found to be optically inactive, confirming the work of Karrer and his associates (7).

TABLE II

*Optical Rotations of Leaf Carotenes Reported by Other Investigators*

Experiment No.	Source of sample	Solvent	Wave-length	Specific rotation	Investigator	Reference No.
			$\lambda$ .	degrees		
1	Horse-chestnut	Benzene	6438	+90	Kuhn and Lederer	4
2	Spinach (winter)	"	6438	0	" "	4
3	Nettles	"	6438	0	" "	4
4	Grass	"	6438	0	" "	8
5	Tea	CS <sub>2</sub>	6563	+370	Yamamoto and Muraoka	9
6	Carrot	Benzene	6678	+37	Mackinney and Milner	10

A summary of the results obtained by other investigators is shown in Table II. It is worthy of note that tea leaves contain almost pure  $\alpha$ -carotene (9) and that carrot leaves in common with carrot roots contain a mixture of  $\alpha$ - and  $\beta$ -carotenes (10).

*Method of Determining Optical Rotations*—The sample of pigment was dissolved in 15.0 cc. of benzene. This solution was introduced into an all-glass polarimeter tube ( $10.0 \times 1.3$  cm.), the ends of which were fused on, were optically plane and parallel, and did not rotate the plane of polarized light. All measurements were made with a Winkel-Zeiss polarimeter, sensitive to  $0.01^\circ$ , whose zero point was independent of the wave-length of illumination. A hot cathode helium arc (11) served as light source.

Monochromatic light of wave-lengths 5876, 6678, or 7065 Ångström units was isolated from the helium arc by means of the following filters.

Wave-length	Zeiss Monochromat filter	Copper sulfate solution (3 cm )
Å.		per cent
5876	A	2
6678	B	0*
7065	A <sup>+</sup> +B	0

\* In determining the rotation of  $\alpha$ -carotene at 6678 Ångström units, a 1 per cent copper sulfate solution was interposed to eliminate completely the light of wave-length 7065 Ångström units. The difference observed with and without this filter was negligible; therefore, this precaution was not taken in other measurements at this wave-length.

All rotations are reported as specific rotation for benzene solution at  $17$ – $20^\circ$  and the wave-length 6678 Ångström units unless otherwise stated.

#### *Melting Points versus Optical Rotations*

It is seen from Table I that the melting points of carotenes with zero optical activity average about  $180.5^\circ$ . The highly rotatory  $\alpha$ -carotene melts at  $182.5^\circ$ .

The melting points of different samples of carrot root carotene have been found to vary. In order to determine whether or not this variation in melting point depended on the relative proportion of the  $\alpha$  and  $\beta$  forms, as measured by optical activity, a series of measurements on the melting points and optical rotations of various samples was made. The results are plotted as circles in Fig. 1. The curve obtained resembles the typical melting point diagram for a two component system in which solid solutions but no compounds are formed.

Mixed melting points of carotenes with known properties (Table III) were also taken. These are plotted as Points 1, 2, 3, and 4 (Fig. 1) and are seen to fall close to the curve. Since Mixtures 1, 2, and 3, made with carrot root and sunflower leaf carotenes (plotted as Points 1, 2, and 3, Fig. 1) lie on the curve, it is evidence that the optically inactive carotenes from both sources are identical.

When three leaf carotenes were recrystallized together, the mixture showed no appreciable lowering of melting point. The melting points of the carotenes were: sunflower 181.3–181.8°,

TABLE III  
*Melting Points of Synthetic Mixtures of Carotenes*

Mixture No.*	Carotene I			Carotene II			Mixture	
	Type	Melting point	[α] <sub>D</sub> 25	Type	Melting point	[α] <sub>D</sub> 25	Melting point	[α] <sub>D</sub> 25
		°C.	degrees		°C.	degrees	°C.	degrees
4	Carrot root, resolved	177.3	+311	Carrot root	179.0	+23.7	162.6	+181
2	Carrot root, Point 4	162.1	+181	Sunflower leaf	180.0		173.5	+69
1	Carrot root		+156	" "	180.0		173.5	+51
3	" "		+156	" "	180.0		170.1	+71

\* These numbers refer to the points designated in Fig. 1.

chard 180.2–181.3°, cauliflower 181.8–182.5°, and mixture 180.7–181.5°.

In one case  $\alpha$ -carotene was prepared, by fractional adsorption, from a single lot of ordinary carrot root carotene. The properties of samples taken at intermediate stages in the resolution are represented by triangles in Fig. 1. One sample (Point 5) is far below the curve and may indicate that an impurity had been formed during the adsorption process, as suggested by Karrer and Walker (6).

*Method of Determining Melting Points*—In order to obtain consistent melting point data a standard procedure must be adopted. The carotenes were recrystallized from *n*-heptane, either as described under the preparation of  $\alpha$ -carotene or in the solubility



measurements. The crystals were dried in a vacuum over calcium chloride and paraffin. All melting points were taken in a Berl block (12) and the temperature (corrected) at which the carotene ran down the wall of the melting point tube was recorded as the melting point.

### *Solubility versus Optical Rotations*

The solubilities of carotenes with different optical activities are recorded in Table IV. These data show that solid solutions

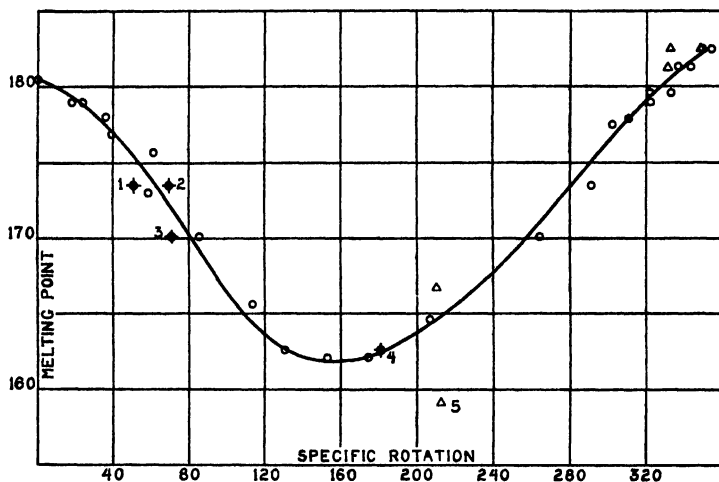


FIG. 1. Melting points of carotenes with different specific rotations. Triangles represent intermediate stages in a single preparation of  $\alpha$ -carotene. Other observations are represented by circles. See Table III for data on Points 1 to 4. Point 5 is explained in the text.

exist, that  $\alpha$ -carotene is slightly less soluble than  $\beta$ -carotene, and that mixtures of the two are much more soluble than either one. A satisfactory explanation of the abnormal solubility of the carotene in Experiment 3 is still lacking.

In Table V some data on the solubilities of mixed carotenes in hexane (from petroleum, Kahlbaum) will be found. It is apparent that the leaf carotenes have identical solubilities. Mixtures of these leaf carotenes with carrot root carotene also have identical solubilities which are intermediate between those of the compo-

nents. This indicates that solid solutions are formed and that leaf and carrot root carotenes have one component in common.

*Method of Determining Solubilities*—The carotene samples were weighed into ampoules, a measured quantity of *n*-heptane added, the ampoules evacuated and sealed. The tubes were heated in a water bath until all of the carotene had dissolved. They were then placed in a thermostat at 25.0° and shaken for 48 to 72 hours.

TABLE IV

*Solubilities in n-Heptane at 25.0° of Carotenes of Different Specific Rotations*

Experiment No.	Specific rotations, $[\alpha]_{25}^D$		Melting point of solid °C.	Solubility mg. per cc.
	Solid phase	Dissolved		
	degrees	degrees		
1	0*	0	180.5	1.22
2	+36	+67	178.0	1.38
3	+153	+147	162.1	4.42
4	+291	+169	173.5	2.20
5	+337	+287	181.3	1.15

\* Sunflower leaf carotene.

TABLE V

*Solubilities of Carotenes in Hexane (from Petroleum) at 25.0°*

Experiment No.	Type of carotene	Melting point of solid °C.	Solubility mg. per cc.
1	Carrot root	176.8	1.12
2	Sunflower leaf	180.2	0.70
3	Spinach leaf	180.7	0.70
4	Carotene of Experiments 1 + 2		1.00
5	“ “ “ 1 + 3		1.00

During this time apparent equilibrium was established between the solution and the solid carotene which separated. The ampoules were removed from the bath, the tops broken off, and the solutions filtered immediately in a closed vessel submerged in the same bath. A measured volume of the filtrate was evaporated to constant weight in a vacuum desiccator. The optical rotations of the crystalline carotene which separated and of the carotene which remained in solution were then measured.

Solubilities of carotene in hexane (from petroleum) were determined in the same way except that the samples were only partially dissolved by heating the solutions to 50° for about 1.5 hours.

It is difficult to obtain reliable solubility data on carotene because of its tendency to form supersaturated solutions. This seems to be particularly true when the carotene in the ampoule has been completely dissolved.

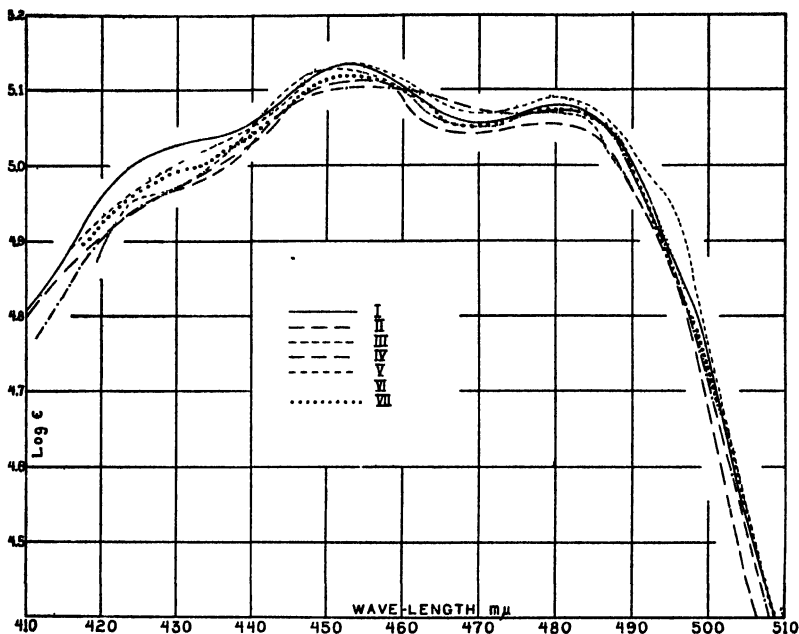


FIG. 2. Absorption spectra of carotenes obtained from different leaves. Curve I represents the results obtained with spinach; Curve II, fig; Curve III, sunflower; Curve IV, chard; Curve V, another sample of chard (Chard 2); Curve VI, alfalfa; Curve VII, cauliflower.

#### *Absorption Spectra versus Optical Rotations*

*Leaf Carotenes*—As will be seen from Fig. 2 the absorption spectra of these leaf carotenes are very similar. There are differences, however, which may be ascribed either to the presence of other carotenoids or to differences in the structure of the carotenes themselves. The carotenes used for these measurements were from the same lots, except Chard 2 (m.p. 183.5°) as previously described (1).

*$\alpha$ -Carotene*—The absorption spectrum of  $\alpha$ -carotene,  $[\alpha] = +354^\circ$ , is plotted in Fig. 3, Curve I; that of the mean for the leaf carotenes in Curve IV. The absorption maxima of  $\alpha$ -carotene are at 4472 and 4755 Ångström units as compared with those of the mean leaf carotene curve at 4529 and 4796 Ångström units.

*Carotenes with Intermediate Rotations*—Absorption spectra of carotenes with specific rotations of  $+316^\circ$  (Curve II) and  $+118^\circ$  (Curve III) are shown in Fig. 3 in comparison with  $\alpha$ - and  $\beta$ -carotene (mean leaf carotene absorption curve). There is a progressive shift in the absorption curve toward the violet as the optical

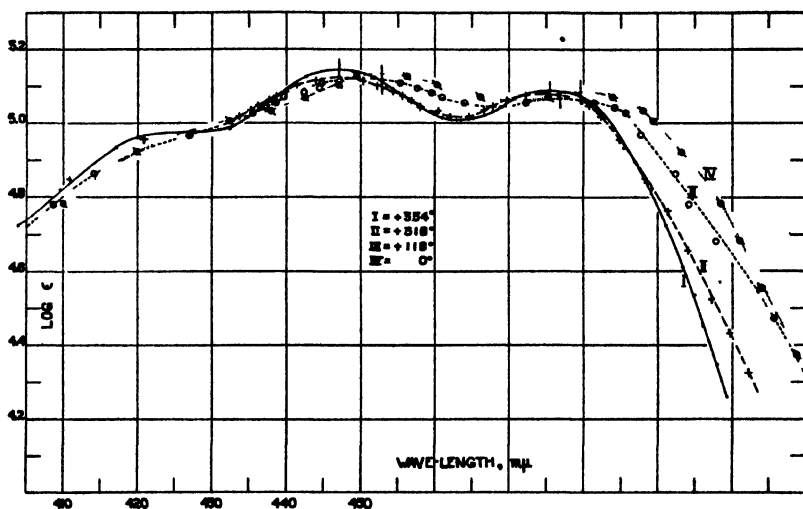


FIG. 3. Absorption spectra of carotenes with different specific rotations

activity of the carotenes increases (8). If these shifts are quantitatively related to the optical activity, theoretical absorption curves can be calculated on the basis of the optical activity of the samples by the equation,  $\log E = \log (xE' + (1 - x)E'')$ , where  $E$ ,  $E'$ , and  $E''$  are the extinction coefficients of the mixture and of the two pure substances respectively and  $x$  is the quotient of the specific rotation of the sample divided by that of  $\alpha$ -carotene.

Calculated curves for samples with specific rotations of  $+47^\circ$ ,  $+118^\circ$ , and  $+316^\circ$  are plotted as solid lines in Fig. 4. The experimental data are represented by circles and are seen to be in fair agreement with the theoretical curves.

**Method of Determining Absorption Spectra**—The solutions used for observation were prepared as follows: About 0.7 mg. of carotene was accurately weighed, dissolved in 0.5 cc. of chloroform, and the solution diluted to 50.0 cc. with 95 per cent ethanol. A further dilution, 1:50, was made with 95 per cent ethanol.

The spectra were photographed on Eastman No. 40 plates, a Bausch and Lomb No. 2750 photometer and No. 2700 spectrom-

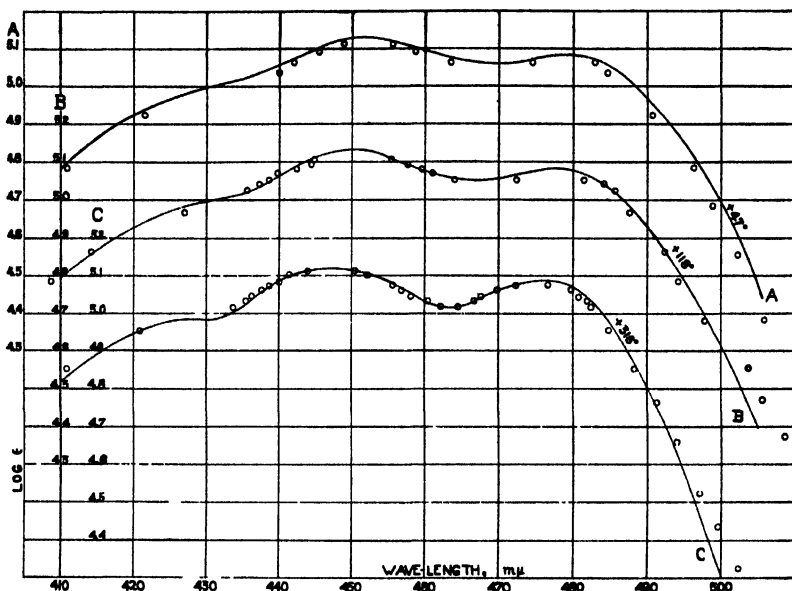


FIG. 4. Absorption spectra of carotenes with different specific rotations. Solid line curve calculated on basis of optical activity; circles, experimentally determined points.

eter and light from a 500 watt Mazda projection lamp being used. The helium spectrum served as wave-length standard.

Near the absorption maxima the plates were measured by means of a photoelectric microdensitometer; all other measurements were obtained by means of a Hartmann wedge visual microphotometer. Both instruments were placed at our disposal through the courtesy of the Mount Wilson Observatory of the Carnegie Institution of Washington.

The molar extinction coefficients were calculated by the usual formula (13).

Near the maxima the calculated errors in  $\log E$  are less than 0.01 unit, in the wave-lengths  $\pm 3$  Ångström units. Toward the bottom of the curve they may be as great as 0.02  $\log E$  unit and  $\pm 10$  Ångström units. When absorption spectra were determined in duplicate the observed differences were found to lie within these limits.

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## STUDIES OF THE RENAL EXCRETION OF CREATININE

### I. ON THE FUNCTIONAL RELATION BETWEEN THE RATE OF OUTPUT AND THE CONCENTRATION IN THE PLASMA\*

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#### INTRODUCTION

The data of Cope (1) on the excretion of creatinine suggest that the output of creatinine, in mg. per minute,  $y$ , may be represented as a linear function of the plasma creatinine concentration,  $x$ , in mg. per 100 cc., of the form

$$y = p + qx \tag{1}$$

where  $p$  and  $q$  are constants.

Cope, from graphic considerations, estimated that for  $y = 0$ ,  $x = 0.5$ . By subtracting 0.5 from every  $x$  and calculating the ratio,  $y/(x - 0.5)$ , he obtained an average value of about 2.07, in the units stated above. From this it would follow that the line fitting his data is  $y = -1.04 + 2.07x$ .

The experiments of Cope differ in several important respects from the earlier experiments of Rehberg (2), and since these differences may influence the results, it will be well to consider them in some detail.

If creatinine is administered by mouth and the concentration of plasma creatinine examined at successive intervals thereafter, it is found that, on plotting on rectangular coordinates with the time as abscissæ, the points representing the plasma concentration seem to lie along a curve which rises rapidly at first, reaches a maximum between 1 and 2 hours after ingestion, and then falls toward the axis of abscissæ. If at the same time the *average* output of creatinine in successive intervals is determined, and the corresponding points are plotted in the same diagram, assuming the

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average to take place at the middle of the interval of urine collection, the points are found to follow a curve analogous to that of the plasma concentration. For the purpose of reference we shall call these curves the  $x$  curve and  $y$  curve, respectively.

Now, Rehberg studied the ratio,  $y:x$ , of points obtained during the downward part of the curves, with a few exceptions to be noted later. Cope, on the other hand, determined the output of creatinine during a single hour, beginning 1 hour after ingestion, and calculated the ratio,  $y:x$ , of the output thus found to the concentration of a single sample of plasma at the middle of the interval of urine collection.

This difference in the obtainment of the points is important from two points of view. In the first place, it is not self-evident that a relation between  $y$  and  $x$  holding during the regular downward part of the curves should also hold during the rise or in the neighborhood of the maximum. In the second place, assuming a unique relationship between  $y$  and  $x$  during the whole experiment, the method of Cope makes use of a critical part of the curve without furnishing the means to avoid systematic errors.

We have carried out a number of experiments on several normal and pathological subjects in an attempt to determine the equation of the whole set of events, both for  $y$  and  $x$ , in terms of time ( $t$ ). The equations are of the general form

$$y = y_0 + ae^{-at} \cdot f(t) \quad (2)$$

$$x = x_0 + be^{-\beta t} \cdot \varphi(t) \quad (3)$$

where  $y_0$  and  $x_0$  are the endogenous values of  $y$  and  $x$  respectively,  $e$  is the base of natural logarithms,  $a$ ,  $b$ ,  $\alpha$ , and  $\beta$  are positive constants, and  $f$  and  $\varphi$  are functions of  $t$  as yet not completely determined. It is known, however, that  $f$  and  $\varphi$  reach asymptotically the value 1 soon after the maximum. This means that, for the decreasing part of the curves, Equations 2 and 3 can be reduced to

$$y = y_0 + ae^{-at} \quad (4)$$

$$x = x_0 + be^{-\beta t} \quad (5)$$

This simplification is of practical as well as theoretical importance, because, once the constants have been calculated, the elimination of  $t$  between pairs of simultaneous equations yields the desired relation between  $y$  and  $x$ ,

The result of the elimination of  $t$  from Equations 4 and 5 is

$$y - y_0 = A(x - x_0)^k \quad (6)$$

where  $A$  is defined by

$$\log A = (\beta \cdot \log a - \alpha \cdot \log b) / \beta \quad (7)$$

$$\text{and} \quad k = \alpha / \beta \quad (8)$$

Equations 4 and 5 show that, after the initial disturbance produced by the rapid absorption of creatinine and the distribution of creatinine in the tissue juices, both the plasma concentration and the rate of excretion of the substance decrease along exponential curves asymptotic to their respective preingestion levels.

One of the advantages of Equations 4 and 5, among others to be discussed later, is that of eliminating the troublesome question as to whether there is true creatinine in the plasma or not; because, regardless of the true chemical significance of the color developed in plasma filtrates by the method of Folin and Wu (3), it is obvious that by this method no amount of ingested creatinine can be detected below this level. In other words, the end-point of the phenomenon is not zero, but a positive quantity equal to the value of the blank analysis.

An objection that could be raised here is that the quantity that has been called  $x_0$  may not have the additive property demanded by Equation 5. This objection, however, can be tested experimentally. The results of two experiments will be given here.

To six pairs of tubes containing a fixed amount of plasma there were added varying amounts of creatinine. To four other tubes no creatinine was added. The tubes were numbered by an independent person, to avoid bias.

*Experiment 1, January 19, 1933*—The total chromogenic value in the plasma filtrate (1:10 dilution), the mean of four separate determinations, was 0.0048 mg. in 5 cc. of filtrate; the standard error of the mean, 0.00067966. The excess of total chromogen over the known amounts of creatinine added to the filtrate, the mean of twelve determinations, was 0.0026; standard error, 0.0006947. The difference between the means was 0.0022; standard error of difference (Fisher's method (4)), 0.0012840; degrees of freedom, 14.

*Experiment 2, February 20, 1933*—The total chromogen in the filtrate, the mean of four separate determinations, was 0.0070;

the standard error, 0.0002653. The excess of total chromogen over the known amount of creatinine added to the filtrate, the mean of twelve determinations, was 0.0064; standard error, 0.00025017. The difference between the means was 0.0006; standard error of difference, 0.0004656; degrees of freedom, 14.

Although the difference of the means in Experiment 1 is relatively large, this difference appears quite insignificant when tested by the method of Fisher (4). In Experiment 2, the difference is also statistically insignificant. We feel justified, therefore, in retaining the assumption of the additive property of  $x_0$  (and *a fortiori* of  $y_0$ ) as used in Equation 5.

In this paper, the data on one normal subject only will be given. But before showing the result of Equations 4 and 5 as applied to these data, another computation on the same data and on the data of Rehberg will be presented, assuming the equation of a straight line.

#### EXPERIMENTAL

The protocols are given in Table I. The time of urine collection was recorded to the nearest minute at the end of micturition. The volume of urine was determined to within 0.5 cc. Toluene was added as a preservative and the samples were placed in the refrigerator until the time of the analysis.

Blood was drawn from the arm veins and the time recorded to the nearest minute at the end of the blood collection. At least 10 cc. of blood were removed each time. The blood was oxalated, centrifuged as soon as possible, and the plasma separated. The tubes with plasma were put in ice water and then in the refrigerator (4-6°) until the time of the analysis.

For the determination of creatinine in the urine we used Folin's colorimetric method (5) with pure creatinine standards. If the creatinine concentration was high, the sample was diluted with distilled water. For the creatinine in plasma the Folin-Wu procedure was used. At least seven color readings were averaged and the average value was corrected from an empirical curve constructed after Gaebler's procedure (6). The curves were redetermined with every new lot of picric acid, and often several times with the same lot. The Klett biocolorimeter was used with direct north light. The determinations were carried out in duplicate,

**TABLE I**  
**Data on Creatinine Experiments**

Experimental details	Time of urine collection	Urine volume	Urine creatinine	Time of blood collection	Plasma creatinine
		cc.	mg. per 100 cc.		mg. per 100 cc.
Experiment E-2, Apr. 29, 1932. 3 gm. creatinine by mouth, 9.55 a.m.; breakfast with coffee, 7.15 a.m. Single determinations of blood creatinine. Routine laboratory work during this and succeeding experiments	8.12-9.06	193	16.2	9.26	1.34
	9.06-9.52	20	165	10.46	5.03
	9.52-10.54	32	834	11.50	6.07
	10.54-11.55	37.5	1500	1.52	3.32
	11.55-1.38	49.5	1505	4.17	1.85
	1.38-3.30	110	432	6.08	1.38
	3.30-4.43	29	692		
	4.43-5.50	28	502		
Experiment E-3, Aug. 12, 1932. 3 gm. creatinine and 20 gm. urea by mouth, 8.41 a.m.; breakfast with coffee, about 7.15 a.m.	7.42-8.38	52	64.8	8.36	1.15
	8.38-9.46	300	66.7	9.40	3.30
	9.46-10.55	131	286.5	10.51	3.55
	10.55-12.07	146	236.5	12.00	3.25
	12.07-1.29	264	135.5	1.22	2.65
	1.29-2.39	293	65.6	2.27	2.20
	2.39-3.43	71	224.0	3.35	1.78
	3.43-4.56	86	167.0	4.47	1.60
Experiment E-4, Nov. 16, 1932. 8 gm. creatinine, 9.39 a.m.; breakfast with coffee about 7.15 a.m.	8.26-9.37	220	25.2	9.31	1.15
	9.37-10.57	203	510	10.53	10.54
	10.57-12.05	67	2080	11.58	10.43
	12.05-12.57	57	1531	12.52	8.07
	12.57-2.14	84	1085	2.06	5.90
	2.14-3.18	93	620	3.13	4.38
	3.18-4.19	22.5	1657	4.09	4.24
Experiment E-5, Jan. 24, 1933. 5 gm. creatinine and 20 gm. urea, 9.01 a.m.; breakfast, 7.15 a.m.	7.56-8.59	348	16.5	8.34	0.97
	8.59-10.16	288	220	10.10	6.56
	10.16-11.12	95.5	702	11.05	6.50
	11.12-12.07	66.5	782	12.00	5.70
	12.07-1.20	153	369	1.12	4.14
	1.20-2.33	88	495	2.23	3.09
	2.33-3.40	54	550	3.38	2.81
	3.40-4.58	53.5	496	4.51	2.41
	4.58-5.59	35	493	5.54	2.00
Experiment E-6. Feb. 8, 1933, 8.20 p.m., 50 gm. casein. Feb. 9, 7.25 a.m., 50 gm. casein; 10.15 p.m., 100 gm. casein. Feb. 10, 7.25 a.m., 50 gm. casein; 7.35 a.m., breakfast; 9.09 a.m., 5 gm. creatinine and 20 gm. urea. Data of Feb. 10	5.59-7.11	38	410	7.05	1.46
	8.14-9.05	325	11.9	9.00	1.00
	9.05-10.21	350	155.5	10.14	6.72
	10.21-11.21	167	477	11.15	6.45
	11.21-12.37	147	537	12.25	5.36
	12.37-1.41	109.5	425	1.34	4.12
	1.41-2.48	103	329	2.40	3.01
	2.48-3.51	80	309	3.43	2.75
	3.51-4.58	74	273.5	4.52	2.56
	4.58-5.47	44.5	279.5	5.36	2.11

TABLE I—*Concluded*

Experimental details	Time of urine collection	Urine volume	Urine creatinine	Time of blood collection	Plasma creatinine
		cc.	mg. per 100 cc.		mg. per 100 cc.
Experiment E-8, July 26, 1933. 5 gm. creatinine, 9.23 a.m.; breakfast, 7.15 a.m.	8.27- 9.21	37	106.0	9.17	0.61
	9.21-10.25	39	1073	10.22	6.65
	10.25-11.28	38.5	1785	11.24	7.16
	11.28-12.28	37.5	1537	12.24	6.17
	12.28- 1.28	34	1234	1.24	4.93
	1.28- 2.29	29	1287	2.25	4.45
	2.29- 3.32	27	1269	3.28	2.97
	3.32- 4.33	20	1111	4.24	2.35

sometimes in triplicate, and not more than five with the same set of standards. The readings were made between 10 and 20 minutes after the addition of the alkaline picrate, the lapse of 10 minutes being consumed by the readings of five samples, with change in standards. The samples of plasma and urine were renumbered by an independent worker and a record kept of the new numbers, in order to avoid bias in the analysis.

The determinations were made in both plasma and urine from 12 to 72 hours after the beginning of the experiment. Repeated checks on plasma, urine, and plasma filtrates, after standing up to 72 hours in the refrigerator, have shown no demonstrable change in the creatinine concentration.

### Computations

*Method 1* ( $y = p + qx$ )—Following the suggestion of the opening paragraph of this article, straight lines were fitted to the data on our Subject E and to the published data of Rehberg on himself (2). The points were paired according to the method of Rehberg, that is, the average rate of output ( $y$ ) was calculated as usual and the corresponding plasma concentration was interpolated graphically (linear interpolation) in a time-concentration diagram.

From the pairs of points, the constants  $p$  and  $q$  were then calculated by least squares. The results are given in Table II, and graphically in Figs. 1 and 2.

Our experiments will be identified as Experiments E-2, E-3,

etc. (Experiments E-1 and E-7 are irrelevant). Those of Rehberg will be designated Experiments R-1, R-2, etc., in the order given in his publications.

Since we are concerned only with the descending part of the curve, no point has been used from our data which was not clearly beyond the maximum. A similar criterion applied to Rehberg's experiments would have omitted the first point of Experiment R-3 (1.19 hours after ingestion of creatinine) and the first plasma value of Experiment R-4 (1.18 hours after ingestion). The first point is indicated by an arrow in Fig. 1. In Experiment R-4, an

TABLE II  
*Straight Lines,  $y = p + qx$ , Fitted to Experimental Points*

Experiment No.	$p$	$q$	Variance*	No. of observations
E-2	-0.8560	1.7014	0.2856	5
E-3	-1.3724	1.8913	0.0752	6
E-4	-1.8104	2.0216	0.6399	5
E-5	-0.8111	1.7609	0.1195	8
E-6	-1.5649	1.8575	0.2736	7
E-8	-0.0722	1.3995	0.2108	6
R-1	-3.6412	1.9851	0.2555	7
R-2	-2.0112	1.6137	0.5358	9
R-3	+2.3540	0.8022	0.7494	6
R-4	-4.2417	2.1371	1.5562	12
R-4 (reduced)	-3.0729	1.8990	0.2251	5
R-5	-1.5685	1.5636	0.1442	8

\* The variance has been corrected for the number of constants determined.

experiment that lasted almost 6 hours, Rehberg managed to get twelve  $y$  values, some of the intervals being as short as 16 minutes. The scattering of the points is quite marked (see the large value of the variance). Whatever the causes of this scattering, a better line is obtained if the intervals are enlarged by grouping into five successive intervals of 77, 68, 61, 72, and 80 minutes, similar, consequently, to the intervals of our experiments. The result of this grouping is shown in Experiment R-4 (reduced), Table II. Notice the marked reduction in the variance.

The real difficulty in the interpretation of the results of fitting

straight lines to these data lies in one of the assumptions of the method used; namely, that the independent variable (here  $x$ ) is free from errors. This assumption being unwarrantable in these experiments, the statistical significance of the differences in the

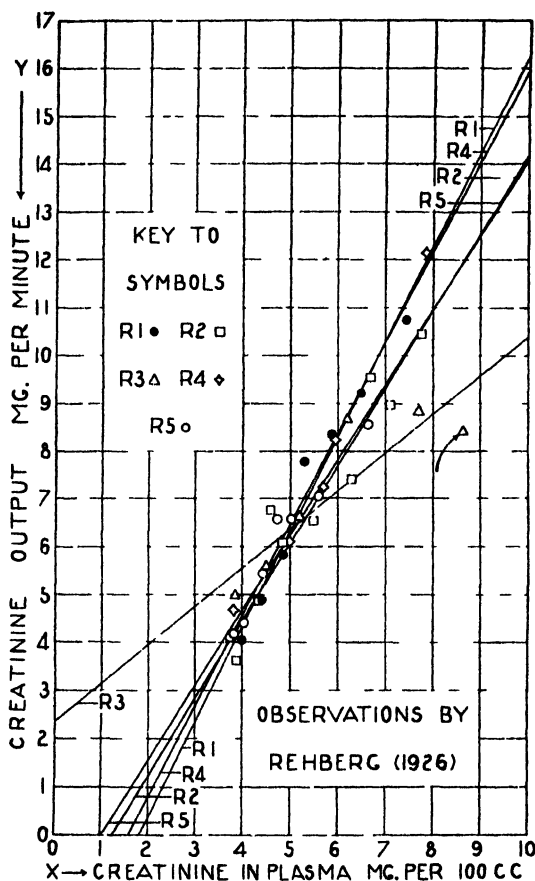


FIG. 1. Straight lines fitted to the data of Rehberg. The numerical values of the constants are given in Table II. Each experiment is given a number, R-1, R-2, etc., in the order of publication. The points from each experiment are identified by different symbols. The first observation of Experiment R-3, marked by an arrow, should have been omitted from the calculation since it was made too soon after the ingestion of creatinine (see text). The points of Experiment R-4 have been grouped by us.

values of the constants cannot be determined directly from these calculations. A better way is to disentangle the errors of observation by fitting suitable interpolating functions to the  $y$  and  $x$  curves, independently of each other. By so doing, the difficulty

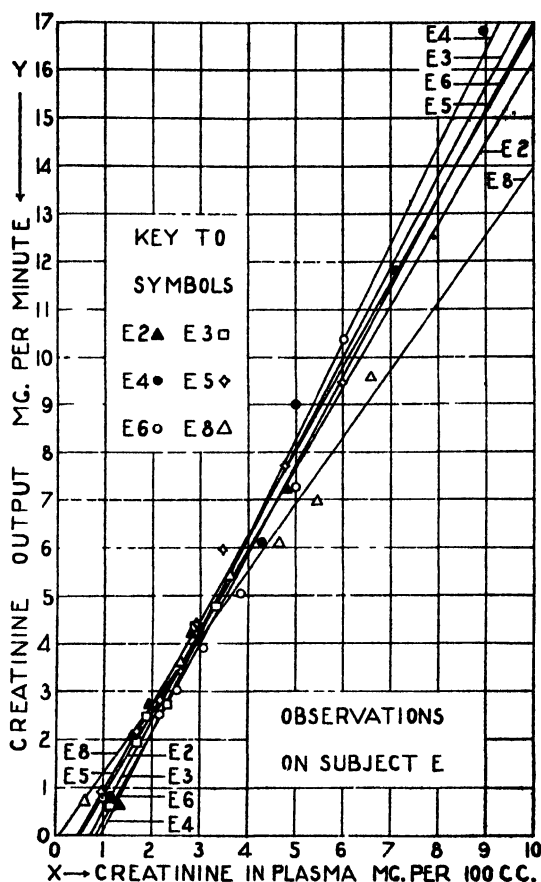


FIG. 2. Straight lines fitted to the experiments on our Subject E. The constants are given in Table II. Each experiment is given a number, E-2, E-3, etc., in the order of Table I. The points from each experiment are identified by different symbols.

referred to disappears, because, the interpolating functions being functions of the time, we can assume the errors in time to be



negligible in comparison with the errors in  $y$  and  $x$ . Before proceeding with this calculation, several suggestions which arise from the data in Table II will be mentioned, to facilitate future discussions.

The slope of the lines for Subject E varies less than that for Rehberg, owing in large measure to the greater range in the values of  $y$  and  $x$  from which the lines were determined. The inclusion of the endogenous points in our experiments, while changing the slope of some of the lines, can be shown not to alter the total variation. By recalculating the curves for Subject E, without the endogenous points, we get, in the same order as in Table II,  $a = -0.257, -1.003, -2.533, -0.822, -2.078$ , and  $+0.0425$ ,  $b = 1.5559, 1.7562, 2.1251, 1.7634, 1.9729$ , and  $1.3769$ .

The largest discrepancy in slope occurs in Experiments E-8 and R-3, both of which were conducted at low diuresis (mean diuresis 0.48 and 0.59 cc. per minute respectively).

If we assume that the scattering of the points in Figs. 1 and 2 is due to errors of observation alone, and calculate the lines fitting all the points from each subject (with the exception of the first point in Experiment R-3, for the reason already indicated), using the method of least squares, the following equations are obtained:

$$\text{Subject E (37 points)} \quad y = -1.174 + 1.807x \quad (9)$$

$$\text{Rehberg (34 " )} \quad y = -1.766 + 1.610x \quad (10)$$

$$\text{Cope's data (21 " )} \quad y = -1.127 + 2.118x \quad (11)$$

The equation of the line fitting Cope's data, calculated by the same method, is given for the purpose of comparison.

Equations 9 to 11, having been derived without regard to differences in the individual experiments, will be looked upon, as far as Rehberg and Subject E are concerned, as rough first approximations to the yet unknown relation between  $y$  and  $x$ . From this standpoint Cope's data and ours may be said to confirm the experimental facts contained in the papers of Rehberg which we have analyzed. That the straight line does not pass through the origin, as perceived by Cope, is, of course, shown by this method of calculating our own and Rehberg's data. This fact, as will be discussed later, removes all significance from the ratio  $y:x$ .

*Method 2* ( $y = y_e + ae^{-ax}$ ,  $x = x_e + be^{-bx}$ )—The endogenous  $x$ ,  $x_e$ , mean of five determinations, is 1.0366 mg. per 100 cc. of

plasma, standard error 0.1009. The endogenous rate of creatinine excretion,  $y$ , mean of six determinations, is 0.7243 mg. per minute, standard error 0.0424. The steps in the calculation of the constants will be merely indicated. The time of the  $y$  determinations, is as before, the mid-point of urine collection, whereas the time of the  $x$  determinations is the time of blood withdrawal. Since these times do not coincide, the fact should be carefully borne in mind and corrected before the elimination of  $t$ . The logarithms of  $y$  and  $x$  are plotted with the time as abscissæ, in order to exclude the early points that do not belong in this logarithmic phase. This determines the zero time for each curve. The equations are then put in linear form by taking logarithms and the constants computed by least squares, the residuals being weighted (Scarborough (7)). The equations fitted are

$$\log (y - 0.7243) = \log a - (\alpha \cdot \log e) t \quad (12)$$

$$\log (x - 1.0366) = \log b - (\beta \cdot \log e) t \quad (13)$$

Seven place logarithms and a calculating machine were used for the arithmetical work. Seven decimal places were carried throughout and the usual checks applied. After the constants had been calculated, the theoretical values of  $y$  and  $x$  were determined and the residuals computed.

The results are shown in Table III. The mean  $\alpha$  is 0.2865, standard error 0.02004. The mean  $\beta$  is 0.2912, standard error 0.02282. The value of  $k$  (Equation 8) from these means is, therefore, practically equal to 1. The elimination of  $t$  from pairs of synchronized equations (Equations 12 and 13) yields Equation 6. The constants  $A$  and  $k$  are set forth in Table IV. The mean value of  $k$  is 0.9933, standard error 0.0594, showing, as before, that the relation between  $y$  and  $x$  can be taken to be linear on an average. The mean value of  $A$  is 1.8177, standard error 0.1204. Writing Equation 6 with this value of  $A$  and  $k = 1$ , we have

$$y - 0.7243 = 1.8177 (x - 1.0366) \quad (14)$$

$$\text{or} \quad y = -1.1599 + 1.8177x \quad (15)$$

the latter equation holding for  $x$  equal to or larger than the endogenous value. Equation 15 should be compared with Equation 9 and the closeness of the numerical value of the constants noted. The fact that Method 1 yields, under certain circumstances, values

sufficiently close to those resulting from a more exact, but laborious, method, will be utilized in the analysis of our data on a number of human subjects, to be published later.

It should be pointed out that if the constant terms of Equations 4 and 5 are omitted, that is, if the data are fitted to exponentials

TABLE III  
*Exponentials Fitted to Excretion Rate ( $y$ ) and Plasma Concentration ( $x$ ) of Creatinine*

$y = y_0 + ae^{-\alpha t}$					$x = x_0 + be^{-\beta t}$			
Experiment No.	$a$	$\alpha$	Variance*	No. of observations	$b$	$\beta$	Variance*	No. of observations
E-2	6.5136	0.3468	0.0005	4	5.044	0.4018	0.0039	4
E-3	4.3316	0.2418	0.1314	5	2.253	0.2822	0.0052	5
E-4	16.008	0.3156	0.1669	4	9.253	0.2859	0.1063	5
E-5	11.2372	0.2494	0.0228	8	5.589	0.2629	0.0474	8
E-6	9.6030	0.3283	0.0240	6	4.228	0.2670	0.0465	6
E-8	8.6679	0.2370	0.2551	5	6.336	0.2476	0.1677	6

\* The variance has been corrected for the number of constants determined.

TABLE IV  
*Resultant Equation,  $(y - 0.7243) = A(x - 1.0366)^k$ , after Elimination of Time ( $t$ ) between Pairs of Corresponding Exponentials*

Experiment No.	$A$	$k$	$\bar{V}$	Standard error of $\bar{V}$	Additional substance ingested
E-2	2.2331	0.8633	0.57	0.1386	Urea
E-3	1.9211	0.8568	2.34	0.5976	
E-4	1.6317	1.1040	1.00	0.2274	
E-5	2.0127	0.9486	1.10	0.2002	" and casein
E-6	1.4149	1.2295	1.41	0.1577	
E-8	1.6928	0.9574	0.48	0.0519	

$\bar{V}$  = mean of urine rates for whole experiment, in cc. per minute.

asymptotic to zero, the numerical results are entirely different. The mean  $\alpha$  becomes 0.2442, mean  $\beta$  0.1976. Similar calculation on the data of Rehberg gives a mean  $\alpha$  0.1804, mean  $\beta$  0.1453. In either case, the value of  $k$  (Equation 8) is about 1.24. The resultant equation,  $y = Ax^{1.24}$ , with different values of  $A$  for Rehberg and Subject E, would give impossible values to the rate

of output at plasma concentrations at all large. This resultant equation, furthermore, does not satisfy the endogenous values of Subject E.

### *Analysis of Errors*

If we make use of the result of the second method of computation, namely that  $\bar{a} = \bar{\beta}$ , all the data on Subject E can be synchronized. The steps will be indicated.

From

$$\log (y - y_e) = \log a - (0.29 \log e) t \quad (16)$$

$$\log (x - x_e) = \log b - (0.29 \log e) t \quad (17)$$

it follows that, since  $t$  and the left-hand sides of Equations 16 and 17 are known, the mean value of  $\log a$  and  $\log b$  can be obtained. From these mean values, say  $\overline{\log a}$  and  $\overline{\log b}$ , the theoretical  $y$  and  $x$  ( $Y$  and  $X$ , respectively) can be calculated from the relations

$$\log (Y - y_e) = \overline{\log a} - (0.29 \log e) t \quad (18)$$

$$\log (X - x_e) = \overline{\log b} - (0.29 \log e) t \quad (19)$$

The residuals  $(y - Y)$ ,  $(x - X)$ , here called errors, are then obtained by returning to the exponential forms of Equations 16 and 17 and Equations 18 and 19.

*Errors of  $x$* —The first calculation of the  $x$  residuals gave a standard deviation = 0.38406. One of the residuals, the first of Experiment E-2, was +1.67, that is, over 4 times the standard deviation, or over 6 times the probable error. This observation should be rejected if the criterion of Wright and Hayford (8) is adopted. Recalculating the residuals after the adjustment of the remaining three observations of Experiment E-2, we get, for a single observation,

Average error.....	0.2288
Standard deviation (s).....	0.2718
Probable error (p.e.).....	0.1833
Positive errors.....	15
Negative ".....	18
No. within $\pm$ p.e. 19.....	expected 16.5, proportionately
" outside $\pm$ " 14.....	" 16.5
" within $\pm$ s, 25.....	" 22
" outside $\pm$ " 8.....	" 11
" within $\pm$ 2s, 31.....	" 31.5
" outside $\pm$ 2s, 2.....	" 1.5

The mean is  $+0.03$ , with standard error =  $0.0473$ , so that the mean is not significantly different from zero.

Owing to the small number of observations, the agreement with the normal distribution of errors is as good as is to be expected, although the number of negative errors is perhaps somewhat large in comparison with the positive errors. The magnitude of the errors can be shown graphically to be independent of the concentration, and since the method yields absolute residuals, this means that the relative error in the determination of the concentration of creatinine in the plasma is larger the smaller the concentration. This is of course reasonable. It should be pointed out that the standard deviation of the endogenous concentration is  $0.2472$ , the probable error of a single observation  $0.1667$ , and the average error  $0.1766$ , in fair agreement with the above determinations from the mean curve, as far as the standard deviation is concerned.

*Errors of  $y$* —The situation is more complicated with regard to the  $y$  curve. The first calculation of thirty-three residuals showed the first observation of Experiment E-5 and the first of Experiment E-8 to yield residuals much too large. Reexamination of the protocols in Table I disclosed the fact that these two points were obtained 1.717 and 1.558 hours, respectively, after the ingestion of creatinine. The large deviations are, therefore, a consequence of the insufficient damping of the function  $f(t)$  of Equation 2, and cannot be counted as errors in the sense of this section. That these deviations could not be detected in the logarithmic line is clear, because "an error in a logarithm may cause a disastrous error in the antilogarithm" (see Scarborough (7), p. 16). These two observations were discarded and the remaining residuals of the corresponding experiments redetermined. The results from thirty-one residuals are, for a single observation:

Average error.....	0.3426
Standard deviation ( $s$ ).....	0.4984
Probable error (p.e.).....	0.3382
Positive errors.....	16
Negative " .....	15

If the absolute errors are plotted, irrespective of sign, in three diagrams, with the following abscissæ: (1) rate of creatinine output, (2) concentration of creatinine in the sample, and (3) rate of

urine flow, there is found a distinct association in (1) and (2). That is, the larger errors are associated with the larger outputs and with the larger concentrations. There is no obvious association with the rate of urine flow.

Consequently, the figures given above for the total error are of little practical significance. The only thing we can do, since the number of observations does not allow of a finer grouping, is to divide the whole range into two parts, from diagram (1), and calculate the error for each separately. For  $y$  between 1.5 and 4.5 mg. per minute (mean = 3.086, fifteen observations) the standard deviation is 0.1725 and the average error of a single observation 0.1274, and for  $y$  larger than 4.5 mg. per minute (mean = 8.150, sixteen observations) the standard deviation is 0.6978 and average error of a single observation 0.5445. In keeping with the above, the determinations of the endogenous output (mean 0.7243, seven observations) give a standard deviation equal to 0.1122 and an average error of a single observation equal to 0.0793.

The complexity in the errors of  $y$  arises in all likelihood from the complexity of the operations to determine it. Three main errors are involved: (1) the errors in the concentration of creatinine in the sample, more or less analogous to the errors in the creatinine concentration of the plasma; (2) errors in the volume of urine, not precisely in the volume of urine voided, but due to the fact that the urine voided is an *estimate* of the amount of urine formed in a given interval; and (3) errors in fitting, since the output is given by an area, but the curve is fitted to loaded ordinates at the middle of urine collection. Furthermore, the errors in (1) and (2) are correlated, owing to the fact that any error in the volume of urine, arising from insufficient emptying of the urinary system, will introduce an error in the concentration of subsequent urine by mixing portions of unlike concentration.

Fig. 3 shows graphically practically all the information that we have obtained so far. The mean lines for  $y$  and  $x$  with their asymptotes have been drawn, and the experimental points plotted according to the method of this section. The original points of Experiment E-2 have been figured so as to show the large discrepancy of the first observation (marked in the figure by an arrow). The ratio of any point on the  $y$  line to the point on the  $x$  line along

the same ordinate is not constant. The ratio of any point on the  $y$  line, measured from its asymptote, to the corresponding point on the  $x$  line, measured from its corresponding asymptote, is constant,

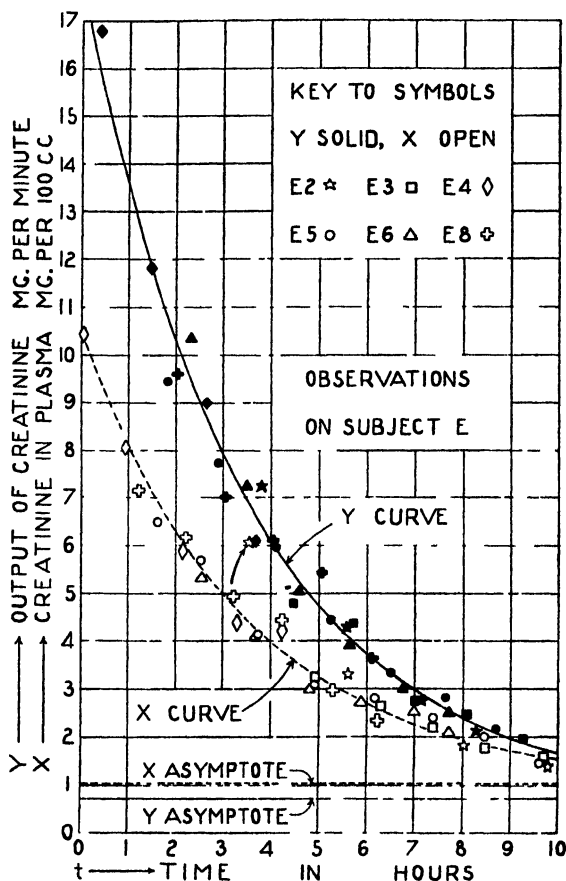


FIG. 3.  $y = 0.724 + 17.202e^{-0.291t}$ ,  $x = 1.037 + 9.463e^{-0.291t}$ . The asymptote of each exponential is shown in the figure. The method of synchronizing the experimental data is given in the text. The time as given has no reference to the ingestion of creatinine. The erratic observation of Experiment E-2 is indicated by an arrow.

thus  $(y - 0.7243)/(x - 1.0366) = (17.202 e^{-0.291t})/(9.463 e^{-0.291t}) = 1.8177$ . This is precisely the content of Equation 14.

Finally, if the deviations from the mean curves are all due to

errors in the determination of  $y$  and  $x$ , and this is the plausible conclusion of the preceding analysis, there is no occasion for other sources of variation arising from the circumstances of the experiments. This implies that the mean values found are independent of moderate exercise, ingestion of urea (Experiments E-3, E-5, E-6), ingestion of casein (Experiment E-6), or changes in diuresis (see Table IV).

### *Summary of Methods of Computation*

Since the methods used here are in no way peculiar to creatinine, but are applicable to any substance that can be determined in the plasma and simultaneously recovered from the urine, it will be in order to add a few remarks on the methods themselves.

The first method, least squares on roughly interpolated data, is not theoretically sound, in that both variables are affected by errors of about equal magnitude. Perhaps the method of Uhler, as given by Scarborough ((7) p. 380), should be used.

The second method, in which exponentials asymptotic to the endogenous level are fitted to the raw data by least squares, is, as far as we can tell, sound both empirically and mathematically. But, in view of the fact that the constants have been calculated from the experimental data the values yielded by the fitted functions, and *a fortiori*, the final values of  $A$  and  $k$ , are dependent upon the errors of observation. In other words, even if the relation between  $y$  and  $x$  were unique for any one individual, it is not to be expected that the most refined method of computation on the best experimental data would give always the same values for  $A$  and  $k$ . This should be carefully borne in mind before the discrepancies in the values of the constants are explained by variation in glomerular activity, changes in posture, diuresis, etc.

### DISCUSSION

In the case of creatinine the results of the third method on data from one human subject show that  $k$  has a mean value equal to 0.99 and  $A$  a mean value of 1.82; that is, that the relation between  $y$  and  $x$  is linear, on an average, from the endogenous level up to about 9 mg. per 100 cc.

Equation 6 can be written

$$y - y_0 = A(x - x_0) \quad (20)$$



that is, the excretion of *ingested* creatinine is proportional to the plasma concentration of *ingested* creatinine.

This relation has been proved only for the downward portion of the curves. Whether this relation holds for the whole curve will be known when the functions  $f(t)$  and  $\varphi(t)$  of Equations 2 and 3 are determined. The work of Cope will be taken up then.

The first method, applied to the same data, yields an equation (Equation 9) almost identical to the mean equation of the third method (Equation 15). This suggests that, when the number of observations and the range of the variables are not very small, the first method is susceptible of yielding a good approximation to the quantities sought.

A glance at Fig. 1 shows that, if Rehberg had made observations between 2 and 3 mg. per 100 cc. of plasma, the large deviation of Experiment R-3 would have been eliminated, because it is improbable that at this level the output of creatinine would have been in the neighborhood of 4.3 mg. per minute. It would have been more likely closer to 2 than to 3, and this alone would have raised the slope of the line quite markedly.

Differentiating Equations 4 and 5 with respect to time, we get  $dy/dt = -\alpha ae^{-\alpha t}$ ,  $dx/dt = -\beta be^{-\beta t} = -bae^{-\alpha t}$ , from which it follows that

$$dy/dt = (a/b) (dx/dt) \quad (21)$$

This means that the rate of change of the excretion rate of creatinine is proportional to the rate of change of the plasma creatinine concentration. In other words, in Fig. 3, the slope of the tangent to the  $y$  curve is proportional to the slope of the tangent to the  $x$  curve at any time. The integration of Equation 21 gives Equation 20.

Since, from Equations 12 and 13

$$(d/dt) [\log (y - y_0)] = -\alpha \cdot \log e \quad (22)$$

$$\text{and} \quad (d/dt) [\log (x - x_0)] = -\beta \cdot \log e \quad (23)$$

and from Table II, (mean  $\alpha$ ) = (mean  $\beta$ ), it follows that the left-hand side members of Equations 22 and 23 are equal, on an average. This means, in words, that the logarithmic rate of decrease of the excretion rate of exogenous creatinine is, on an average, equal to the logarithmic rate of decrease of the plasma concentra-

tion of the same substance. Inversely, when the latter condition is fulfilled, the relation between the two variables is linear.

A systematic investigation of the urinary constituents and of substances excreted by the kidney, along the lines indicated above, will help to determine their excretion law. But, whatever the method of computation, it is obvious that the ratio cannot be used.

*Ratio, Output of Creatinine to Plasma Creatinine Concentration*—This ratio was introduced by Rehberg as a measure of the rate of glomerular filtration ( $F$ ). In the units chosen by Rehberg,  $F$  is 100 times the ratio  $y:x$  of our symbols. We are not concerned here with the assumptions leading to the formula of Rehberg. We shall simply show that practically all the systematic variation of  $F$  in Rehberg's data is produced by the form of Equation 1 with the numerical value of the constants in Equation 10.

Dividing Equation 1 through by  $x$ , we get

$$y/x = q + (p/x) \quad (24)$$

that is, the ratio is not constant, but varies from a limiting value  $q$  to 0 as  $x$  diminishes from  $-\infty$  to  $x = -p/q$ . (For  $x$  less than  $-p/q$ , the ratio becomes negative.) In the case of Equation 10 we have

$$y/x = 1.610 - (1.766/x) \quad (25)$$

Substituting on the right-hand side of Equation 25 first the largest value of  $x$ , then the smallest, from the protocols of Rehberg, performing the operations indicated, and multiplying the result by 100, to conform with this author's units, we get for  $x = 8.80$ ,  $F = 141$ ; for  $x = 3.46$ ,  $F = 110$ . Perusal of the protocols shows  $F$  to vary between 146 and 97, if we discard two values as follows: 200 (first  $F$ , (2) p. 474), out of our calculations by the grouping of the intervals of Experiment R-4; and 57 (last  $F$ , (2) p. 467), due to an error in the computation of the urine rate. Calculated with 2.04 cc. per minute, instead of 1.26, as given,  $F$  becomes 93.

The variations of  $F$  are, therefore, numerical consequences of Equation 10, and cannot, consequently, represent changes in the rate of glomerular filtration. The remaining variation of  $F$  is easily accounted for by the errors of observation.

It could be asked, whether  $q$  (multiplied by a proper factor, for the sake of homogeneity) is the filtration rate. We shall answer

that the determination of the filtration rate (if there is filtration) is not a mathematical problem, but an experimental operation, at the present time unrealizable. We shall not use or recommend this terminology.

The quantity  $q$ , which is equal to the constant  $A$  in Equation 20 and to the constant  $a/b$  in Equation 21, we shall call the *excretion constant* of ingested creatinine.

Cope called the ratio  $y:x$  (multiplied by a factor due to the units employed), "creatinine clearance," by analogy to the terminology introduced by Möller, McIntosh, and Van Slyke (9) for the urea ratio of Addis, but since the value of the ratio tends to become small toward the endogenous level, he suggested the transformation mentioned in the "Introduction," and called the resulting ratio "corrected clearance."

In the first place, if we remove the points corresponding to a value of  $x$  less than 2.0 mg. per 100 cc. from the data of Cope, and calculate the line anew, we get  $y = -0.933 + 2.083x$ , which shows that the constant  $q$  is not seriously affected by the relatively large errors of the low creatinine concentrations in the plasma. See Equation 11.

In the second place, the transformation of Cope, although leading to a value practically equal to that of the excretion constant, is not identical with our Equation 20. The transformation of Cope consists in writing  $y/(x - 0.532) = 2.118$ , with the values from Equation 11, or  $y = 2.118(x - 0.532)$ .

The value 0.532 is the intercept of the line (Equation 11) on the axis  $y = 0$ , but this value is a meaningless extrapolation, because there is no known condition in which the output of creatinine is zero, except total anuria, and in this case, of course, the functional relation between  $y$  and  $x$  ceases to exist. Cope could have extrapolated to any other value, to the value  $x = 0$ , let us say, and write the "corrected clearance,"  $(y + 1.127)/x = 2.118$ , with the same result as before. It is true that Cope tried to justify his transformation by saying that, "Gaebler has been able to isolate from human blood 0.4 to 0.6 mg. of true creatinine per 100 cc.," but Gaebler (6) concludes the paper quoted by Cope as follows: "Evidence is presented that the creatinine-yielding substance in normal blood, while not creatine, is not creatinine itself." We fail to see how Gaebler's statement justifies Cope's assumption. In

fact, if Gaebler's conclusion is correct, the whole of the endogenous value should be subtracted as a blank figure. But the subtraction of  $x_*$  alone (10), will not suffice, because, if we write  $x'$  for this difference,  $x' = x - x_*$ , then it follows, from Equation 20, that

$$y = y_* + Ax' \quad (26)$$

that is, the equation of a straight line intersecting the  $y$  axis at the endogenous output, a positive quantity. The ratio  $y/x'$ , from Equation 26, namely

$$y/x' = A + (y_*/x') \quad (27)$$

will also show a systematic departure from its limiting value  $A$ , only this time the approach will be from above. Suppose that, in order to give an example, we calculate the ratio (Equation 27) from the data of Rehberg, and assume with him, for the sake of the illustration, that his endogenous output is  $y_* = 1.33$  ((2), p. 450). Then Equation 10 becomes  $y - 1.33 = 1.610 (x - 1.924)$ , that is, written in the form of Equation 26,  $y = 1.33 + 1.610 x'$ , or

$$y/x' = 1.610 + (1.33/x') \quad (28)$$

To the points  $x = 8.80$  and  $x = 3.46$ , as before, there correspond  $x' = 6.88$  and  $x' = 1.54$ . The values of the ratio (Equation 28) are, multiplied by 100, for  $x' = 6.88$ ,  $100 y/x' = 180.3$ , for  $x' = 1.54$ ,  $100 y/x' = 247.4$ .

In other words, from one single line (Equation 10)—and the least squares solution is unique—we can obtain, at the point  $x = 3.46$ , a ratio equal to 110 or 247, according as we use Equation 25 or Equation 28, and this without allowing for the errors of observation.

If we try to estimate  $A$  from Equation 20 by calculating the ratio  $(y - y_*)/(x - x_*)$ , and use the average error as the measure of precision, we get

$$(y - y_* \pm \eta)/(x - x_* \pm \xi) = A' \quad (29)$$

when  $\eta$  and  $\xi$  are the average errors in  $y$  and  $x$ , respectively, and  $A'$  is an estimate of  $A$ .

Substituting in Equation 29 the numerical values from Equation 14 and for  $\eta$  and  $\xi$  the calculated values (see "Analysis of

errors"), we have for  $y = 3.086$ ,  $x = 2.336$ ,  $\eta = 0.1274$ ,  $\xi = 0.2288$ ,  $A' = 2.326$  or  $1.463$ , according to the choice of the signs in Equation 29, that is a deviation of 28 or 20 per cent from the mean value 1.818. For  $y = 8.150$ ,  $x = 5.122$ ,  $\eta = 0.5445$ ,  $\xi = 0.2288$ ,  $A' = 2.066$  or  $1.595$ , that is, a deviation of 14 or 12 per cent from the mean value. If, instead of using the average error, the actual experimental values are used, the results may be worse.

It is to be feared that much confusion will arise from the indiscriminate use of the ratio.

#### SUMMARY AND CONCLUSIONS

It is shown that, after the initial disturbance produced by the ingestion of creatinine, both the rate of output of the substance by the kidney and its concentration in the plasma decrease along simple exponential functions of the time, asymptotic to their respective endogenous levels. The mean logarithmic rates of decrease of these functions are equal, from which the linear relation between the output and the plasma concentration follows quite naturally.

If the endogenous level is the end-point (and the initial point, for that matter) of the phenomenon studied, and the coordinate axes are shifted parallel to themselves, so as to bring the new origin to the endogenous point, the result can be simply expressed, that the output of ingested (exogenous) creatinine is proportional to the concentration of ingested (exogenous) creatinine in the plasma. The constant of proportionality is called *excretion constant of creatinine*.

The excretion constant is independent of moderate exercise, simultaneous ingestion of urea or casein, and of moderate changes in diuresis.

It is demonstrated that, if the exponential functions of the time are made asymptotic to zero, the resultant equation from the same experimental data is not linear. This result is also proved from the data of Rehberg.

The straight line representing the changes in the rate of output corresponding to changes in the plasma concentration does not go through the origin of the coordinate system, if the axes are represented by the zero output and the zero concentration. This fact introduces a systematic variation in the ratio,  $y:x$ .

The method of analyzing the two curves separately provides a mean of determining the errors of observation. These errors account for the remaining variation in the ratio. When the errors of observation are allowed for in the estimate of the ratio, a discrepancy of as much as 28 per cent from its mean value may be obtained from this source alone, and still greater if the actual experimental data are used.

The ratio, therefore, called variously glomerular filtration rate or creatinine clearance, cannot be used to determine the excretion constant of creatinine.

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## PHOSPHATASE STUDIES

### VI. NON-OSSEOUS ORIGINS OF SERUM PHOSPHATASE. ITS INCREASE AFTER INGESTION OF CARBOHYDRATES\*

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The osseous origin of serum phosphatase has undoubtedly been all the more readily accepted (to the virtual exclusion of all other possible origins except the leucocytic origin (2)) because of the increased plasma phosphatase in various bone diseases, first reported in a comprehensive manner by Kay (3). In our opinion the findings in bone diseases indicate the osseous origin of the serum phosphatase increment only (4). Similar reasoning led us to assume the hepatogenous origin of the increased plasma and serum phosphatase found in jaundice (5, 6). That non-osseous factors determine the serum phosphatase content was also indicated by the decrease of serum phosphatase observed after prolonged fasting (7). It would be difficult indeed to conceive a connection between a comparable decrease of plasma phosphatase and processes occurring in the bone during the relatively short fasting periods which sufficed to demonstrate this effect. In view of the occurrence of phosphatase in the intestinal mucosa, liver, kidneys, and other tissues,<sup>1</sup> we assumed that the effects of fasting and diet on plasma phosphatase (7) were due to "the physical and chemical processes associated with nutrition." The proof of the existence of non-

\* A preliminary report based upon the results of Series I was presented before the Twenty-sixth meeting of the American Society of Biological Chemists at Philadelphia, 1932; we noted in the summary that, "specific effects on plasma or serum phosphatase may be found during the period of digestion and absorption" (1).

<sup>1</sup> References will be limited in this paper to the recent reviews by Robison (2, 8) and Kay (9), when these contain further references to other workers reporting similar data.



osseous factors determining the serum phosphatase content was in this case apparently achieved by their suppression or inhibition.

The different diets, used in the earlier experiment for a month or longer, might, however, be conceived to have been responsible for the different plasma phosphatase figures through their different effects on bone metabolism, although the adequate calcium content of both diets employed and the normal appearance of the bones argue against any such assumption. It was therefore desirable to test the effects of specific foodstuffs on serum phosphatase<sup>2</sup> during periods short enough to avoid all possibility of bone involvement. We have found that carbohydrate ingestion raised serum phosphatase, that protein lowered it, and that fat was apparently without effect.

We have thus confirmed the earlier indications of the rôle of non-osseous factors, in this case by their stimulation; the increased serum phosphatase after carbohydrate ingestion is probably to be explained by increased functional activity of the organs and tissues involved in the processes that follow ingestion.

The literature on the relations of carbohydrates and phosphates in the metabolism of either or both is voluminous and the results relevant to this study are well known. Ingestion of carbohydrates, and the injection of insulin, adrenalin, or phlorhizin have been repeatedly shown to be followed by changes in the levels of serum and urinary inorganic phosphates. It has also been well established that hexosephosphates appear as intermediate substances in carbohydrate and phosphate metabolism. The action of phosphatase-phosphatase (catalyzing synthesis and hydrolysis, respectively) is thus implied, and the presence of this enzyme in the intestinal mucosa, liver, and kidneys suggests its rôle in the functions of these organs. While contributions by these organs to serum phosphatase are thus conceivable, they could not, however, be suggested with any confidence until a rise of serum phosphatase could be demonstrated in a conspicuous manner, as

<sup>2</sup> The paper just cited (7) was based on the last of several preliminary studies in which we determined plasma phosphatase. We prefer the serum phosphatase determination for reasons stated elsewhere (7, 10) and have employed it for over 2 years. However, the results of earlier plasma phosphatase studies remain substantially valid; the procedure used (11) was accurate except for the oxalate error.

in the present experiments, under peculiar conditions definitely associated with the functions of these organs.

### *Plan of Experiments*

*Series I*—In a preliminary exploration of suitable means of studying the effects of administration of carbohydrates on serum phosphatase, dextrin was administered by stomach tube to six young dogs (litter mates) at the end of a 6 day fast (15 gm. of dextrin per kilo of body weight, in a total volume of about 250 cc.). Dextrin was chosen in preference to glucose in order to make possible more prolonged observation of the anticipated phenomena: hyperglycemia and hypophosphatemia could be expected to continue during the first 9 hours of the 24 hour period (Fig. 1). This period was followed by a meal of meat.

In another experiment, two 12 kilo dogs were fasted for 72 hours. One received 100 gm. of glucose and the other 100 cc. of 40 per cent cream, each in a total volume of 250 cc. The results were negative. We assumed that the demonstration of the specific effects of carbohydrate ingestion was facilitated by the use of young animals after a prolonged fast.

*Series II*—The strictness of the fast in Series I was placed in doubt, particularly by a smaller loss of weight in some cases than was to be expected. (As it developed subsequently, whatever inconsistencies in detail may have been caused by possible errors in enforcing the fast, the essential features, while not demonstrated with the same remarkable uniformity as in Series II, Period A, were not obscured.) Series II was controlled more carefully. Two test periods were used, Periods A and B. During Period A four dogs about 3 months of age (litter mates) were fasted for 6 days, with free access to water (*F*, Fig. 1). Daily weighing and a consistent decline of serum phosphorus and phosphatase served as checks upon the strictness of the fast. At the end of the fast 100 gm. of dextrin in a total volume of 250 cc. were administered to each of the four dogs (Fig. 1, Series II, Period A). During the succeeding 2 weeks the dogs recuperated fully from the effects of the fast and exceeded their initial weights (before the fast) by about 1.5 to 2 kilos.

\* See asterisk foot-note to title.

Period B of Series II, which followed, was designed as a control upon Period A, the same dogs being used. A fast of 7 days duration was enforced, during which the dogs were managed as in Period A (the initial analyses in this period were performed 48 hours after the last meal). Then one dog received 100 gm. of dextrin, as before; one received 100 gm. of glucose, a third 100 cc. of 40 per cent cream—each in a total volume of 250 cc.; the fourth puppy received 250 cc. of water (Fig. 1, Series II, Period B).

### *Methods*

The dogs were bled from the jugular vein without anesthesia and without resistance or struggle on the part of the dogs—at 24 hour intervals while the animals were fasting, and at 2 and 3 hour intervals after administration of the test meal. Serum was obtained for the inorganic phosphorus and phosphatase determinations by allowing the blood to clot spontaneously, the serum being separated by centrifuging twice, when necessary. The inorganic phosphorus and phosphatase were determined by methods previously described (10–12). Blood glucose was determined by the Folin-Wu method.

### *Results*

*Effects of Dextrin Ingestion*—In Series I<sup>3</sup> serum inorganic phosphorus and serum phosphatase decreased during the preliminary fasting period, but these results were inconsistent, probably because some of the dogs had not been maintained on a strict fast. After ingestion of dextrin, serum inorganic phosphorus decreased for 6 hours; at the 9 hour interval it had returned in two or three dogs to near the initial level, while in others it remained low. Serum phosphatase rose to its maximum value 9 hours after the ingestion of dextrin, with two exceptions; 15 hours later serum phosphatase had declined, with one exception, to within 1 unit of the initial value (Fig. 1, Series I).

In the more strictly controlled Series II, the prolonged fast caused consistent and marked declines of serum inorganic phosphorus and serum phosphatase (shown in  $F_6$  and  $F_7$ , Fig. 1, as straight lines, the deviations not being significant enough to be indicated). Upon administration of dextrin during Period A the decline of serum inorganic phosphorus and the increase of serum

phosphatase were more uniform and more marked than in Series I. The significance of the results in Series II, Period A, seems to be enhanced by this striking uniformity of the effects of dextrin, as well as by the similarity of its effects in the same dog during both

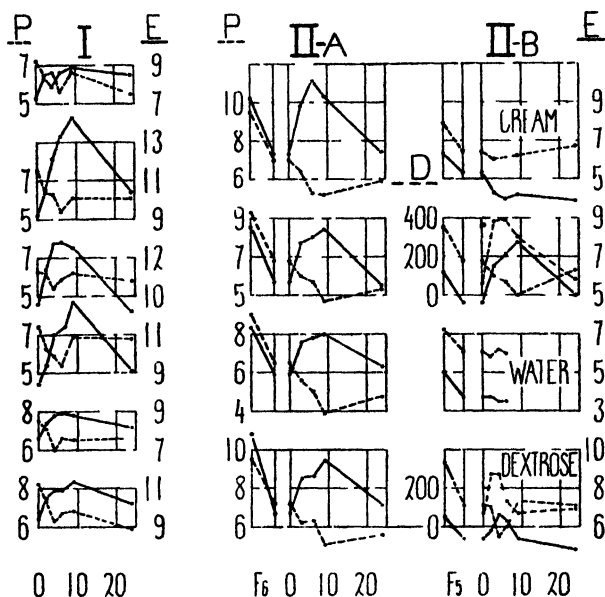


FIG. 1. Effects of ingestion of dextrin on serum inorganic P and serum phosphatase. The roman numbers heading the sections of the chart represent the series; the letters following them, the period designation;  $F_6$  (Series II, Period A), effects of 6 days preliminary fast;  $F_5$  (Series II, Period B), effects of 5 days fast. Controls in Series II, Period B, received cream, water, or glucose, as indicated. The abscissae measure hours after ingestion; the ordinates: P, serum inorganic phosphorus, mg. per 100 cc. (plotted as broken lines); E, phosphatase, units per 100 cc. (solid lines); D, blood glucose, mg. per 100 cc. (dots and dashes). The section for Series I was shown at the Twenty-sixth meeting of the American Society of Biological Chemists at Philadelphia, 1932.

Periods A and B. The rise of serum phosphatase was shown in Period B to be associated with a high blood glucose, the maximum glucose value being reached in advance of the maximum phosphatase value (between 3 and 6 hours after ingestion of dextrin); the maximum serum phosphatase and the minimum inorganic

phosphorus values seemed to be reached at the same time (9 hours after ingestion of dextrin, with one exception), the trend of the one forming a striking counterpart of the trend of the other, sometimes even in minor details. *The rise of serum phosphatase seemed to be most rapid during the first 3 hours after ingestion of dextrin.* Serum inorganic phosphorus, serum phosphatase, and blood glucose had returned to their initial values 24 hours after ingestion of dextrin (Fig. 1, Series II, Period A).

*Controls (Fig. 1, Series II, Period B)*—In view of the probably more rapid completion of the cycle, it was deemed necessary to shorten the intervals between analyses after administration of glucose. It seems that the maximum glucose value was reached between the 2 and 4 hour intervals; the maximum serum phosphatase and the minimum inorganic phosphorus were reached about 4 hours after ingestion of glucose; a trend toward the normal was evident at the 6 hour interval; and substantially initial values were reached at the 9 hour interval; no significant changes occurred during the next 15 hours. *It must be noted that the rise of serum phosphatase was not as marked as after dextrin ingestion.*

40 per cent cream caused no significant change in the serum inorganic phosphorus, and a hardly significant decline of serum phosphatase.

Water showed no significant effect upon serum inorganic phosphorus or serum phosphatase during the first 6 hours; therefore, no further observations were deemed necessary.

*Effects of Meat Ingestion*<sup>3</sup>—24 hours after the ingestion of dextrin in Series I, lean meat was fed to the six dogs and analyses were performed 4 hours later. The results were as follows:

Initial		After 4 hrs		Initial		After 4 hrs.		Initial		After 4 hrs.	
P	E	P	E	P	E	P	E	P	E	P	E
5.5		8.1		6.6		9.6		6.8		8.7	
	8.5		6.7		8.2		7.1		9.1		7.5
5.8		7.6		6.1		6.8		5.9		8.1	
	9.2		8.5		10.4		9.7		10.2		8.9

P represents serum inorganic P (mg. per 100 cc.); E, serum phosphatase (units per 100 cc.).

A sharp increase of serum inorganic phosphorus occurred in all but one case, accompanied by a decline of serum phosphatase,

which was consistent, although in some dogs not as marked as in others. We consider these changes significant not only because they were all in the same direction, but also because they greatly exceeded the diurnal variations (13). Furthermore, they emphasize the specificity of the serum phosphatase increase after carbohydrate ingestion.

#### DISCUSSION

When we were able to demonstrate the effects of glucose, they were essentially the same as those of dextrin; the consistent and more marked results after administration of the latter seem to be due to the longer period of intestinal absorption of glucose after dextrin ingestion. The question arises whether the increase of serum phosphatase following carbohydrate ingestion and the simultaneous and reciprocal fall of inorganic phosphorus (both preceded by the rise of glucose) are related to absorption as such, or to metabolism or storage of carbohydrate, to reabsorption of glucose from the kidney tubules, to the excretion or retention of phosphate, or to any combination of these factors.

Robison (8) grants to the intestinal phosphatase merely a digestive function.<sup>4</sup> The view that the kidney phosphatase may be concerned in the excretion of inorganic phosphorus (16) was seemingly abandoned after Brain, Kay, and Marshall (17) proved that the plasma inorganic phosphorus was the source of the inorganic phosphorus of the urine. Their evidence seemed to involve necessarily the conclusion that the kidney phosphatase was not concerned in the excretion of phosphates. This conclusion need not follow if transitory formation of hexosephosphates in the kidney tubules (as well as in the intestinal mucosa) is assumed, as

<sup>4</sup> Magee and Reid (14) showed that the rate of intestinal absorption of glucose was increased in the presence of phosphate, concluding that there was no evidence of "structural alteration occurring in the glucose molecule, either in the intestinal lumen or after it has passed through the epithelium," and that if "any such compound [a glucose-phosphate compound more diffusible than glucose itself] is, in fact, formed it must be of a very labile nature." Wilbrandt and Laszt (15) suggested the transitory formation of hexosephosphates in the intestinal mucosa during glucose absorption, and explained the effect of phosphates on the rate of absorption by the steep glucose concentration gradient between the intestinal lumen and the interior of the cells of the mucosa, within which the concentration of glucose is diminished through its conversion into the ester,

suggested by Lundsgaard (18) in reports published after the completion of our experiments. Lundsgaard has shown that phlorhizin inhibits the action of phosphatase-phosphatase on the one hand, and absorption of glucose from the intestine on the other. He concludes that the inhibition by phlorhizin of intestinal absorption of glucose is due to the inhibition by phlorhizin of the synthesis of hexosephosphates, the formation of which is a stage in carbohydrate absorption. Having also shown that in phlorhizin diabetes the kidney contains phlorhizin in sufficient concentration to inhibit phosphatase action, Lundsgaard further concluded that phlorhizin glycosuria is due to the inability of the kidney to effect the synthesis of hexosephosphates, which is a stage in the reabsorption of glucose from the kidney tubules. The evidence is suggestive, and the reasoning plausible.

While the phosphatase content of the muscle tissue is low, its total mass may justify its consideration among the sites of increased phosphatase activity after ingestion of carbohydrate, and among the sources of increased phosphatase in the serum. Harrop and Benedict (19) first suggested the retention of phosphorus in the muscle and the formation of an intermediate carbohydrate-phosphate compound after injection of insulin to explain the lowered serum inorganic phosphorus and the increased muscle organic phosphorus (when the muscle was excised before convulsions had set in).

The question also arises to what extent the decrease of serum phosphatase after meat ingestion may be due to the effect of amino acids. Bakwin and O. Bodansky (20) and A. Bodansky (10, 13) have found that glycine inhibited phosphatase activity. It may be suggested that the demonstration of similar amino acid content in the serum is unnecessary, for the effect of meat upon serum phosphatase under our experimental conditions may depend on the inactivation of phosphatase *in vivo*, with the formation of a complex which is adsorbed or otherwise retained without appearing in the serum.

Because of lack of means of differentiating the serum phosphatase or phosphatases by their origin from the various organs, the rôle of any organ or group of organs as a source of serum phosphatase may be considered clearly established when serum phosphatase increase is unmistakably associated with an experimental

or pathological deviation from the normal function of the particular organ or organs. The sum of our information justifies, in our belief, the generalization that *increased serum phosphatase is due in any given case to pathological or functional overproduction or oversecretion of the phosphatase of one or more of several organs and tissues known to contain phosphatase and known to be involved in the given pathological or physiological process.* This generalization may be extended. All changes of serum phosphatase may be considered due to pathological or functional suppression or stimulation of those organs and tissues. Furthermore, this generalization may include the assumption of the diverse origin of normal serum phosphatase as well; it may help explain the wide range over which normal serum phosphatase varies from species to species and from individual to individual within the same species (man and experimental animals, young and adult), while the range is very narrow in the same normal adult individual, or in the young when the determinations in the latter are performed at suitable intervals.

#### SUMMARY AND CONCLUSIONS

1. Alimentary hyperglycemia, and the hypophosphatemia associated with it, are accompanied by an increase of serum phosphatase.

2. The demonstration of this relationship is apparently favored by the employment of young dogs after a preliminary prolonged fast. The use of dextrin prolongs the course of the phenomena observed.

3. Control experiments indicate the specificity of these effects of carbohydrate ingestion. Diurnal variations (after ingestion of water only) are insignificant. Proteins (meat) lower serum phosphatase; fats (cream) have little if any effect.

4. It is suggested that the increase of serum phosphatase after carbohydrate ingestion is due to increased functional activity, with transitory hexosephosphate formation and oversecretion or overproduction of phosphatase by the intestinal mucosa, liver, kidneys, and possibly muscle.

5. The serum phosphatase increase may be considered as of osseous origin in bone diseases, hepatogenous in catarrhal jaundice or hepatitis, and probably of mixed origin (intestinal mucosa, liver, kidney) after administration of dextrin. It is suggested that normal serum phosphatase is of similarly diverse origin.



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# GLUCOREDUCTONE FOR THE STANDARDIZATION OF 2,6-DICHLOROPHENOLINDOPHENOL SOLUTIONS USED FOR THE ESTIMATION OF ASCORBIC ACID (VITAMIN C)\*

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A simple method is presented for the standardization of the indophenol indicator solution which was proposed by Tillmans, Hirsch, and Hirsch (4) for the quantitative titrimetric determination of ascorbic acid. Such indicator solutions change rapidly upon standing and the uncertain end-point in titration when old solutions are used makes it necessary to use fresh solutions, thus requiring frequent standardization with ascorbic acid. This material, however, is not easily available at the present time, and therefore a procedure was worked out by which the titer of the indicator solution may be determined by some other means but may still be expressed in mg. of ascorbic acid equivalent to each cc. of the indicator solution.

A number of investigators observed that when alkaline sugar solutions are heated, decomposition products capable of reducing the 2,6-dichlorophenolindophenol solution are formed. Von Euler and Klusmann (2) made elaborate studies of the formation and characteristics of these highly reducing materials to which they gave the provisional name reductones. Von Euler and Martius (3) reported the isolation of a glucoreductone having a reducing power about 50 per cent higher towards the indophenol indicator than has pure ascorbic acid.

It is not the purpose of this paper to discuss the limitations of the titrimetric method for the estimation of ascorbic acid, which is becoming of general use, as it has been shown that the reducing

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power of fresh fruits and vegetables is due almost entirely to the ascorbic acid present. It has been found, however, that under standardized conditions a constant amount of reductone can be formed from glucose which may be used for the standardization of the indicator solution. The experimental procedure used to obtain the solution of the glucoreductone and proposed as standard is as follows:

5 cc. of a 0.5 per cent solution of Bureau of Standards glucose are placed in a test-tube, 0.5 cc. of 0.5 N NaOH is added, and the tube

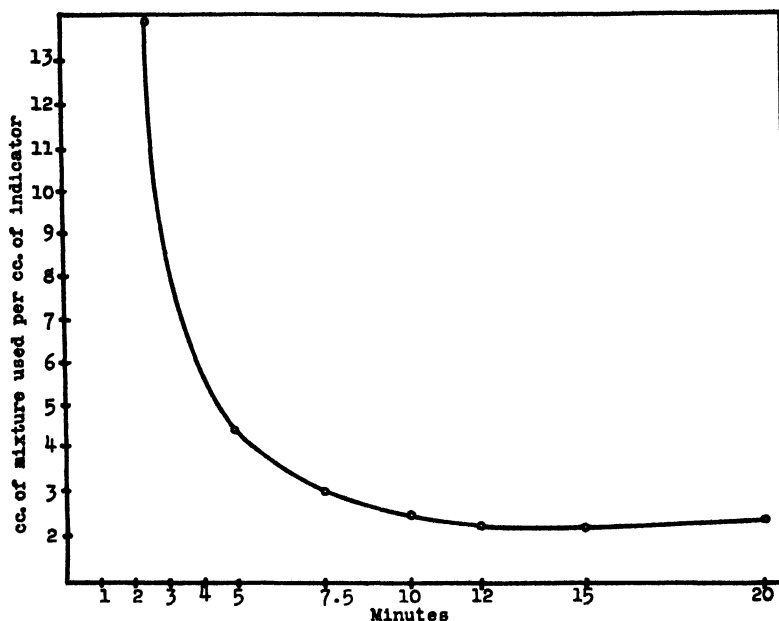


FIG. 1. Reductone formation in heated alkaline glucose solution

closed by a Bunsen valve. The test-tube is immersed in a water bath of 80° for exactly 12 minutes and at the end of this period it is cooled and 1 cc. of 10 per cent HCl added, making a total volume of 6.5 cc. This reaction mixture is used immediately for titrating a small amount (0.1 to 0.5 cc.) of the indicator solution from a microburette according to the Birch, Harris, and Ray (1) modification of Tillmans' method. After the pink color disappears, the solution is divided into 2 equal parts and to 1 part 1 more drop of the reaction mixture is added to make certain that the titration has been

completed. This is necessary because even with fresh indicator solutions, the color often changes to a brownish one, which, however, does not change further when more reductone solution is added and which must be regarded as the end-point.

When in a series of determinations the heating time was varied from 0 to 20 minutes, the amount of reductone found became almost constant after 12 minutes of heating. Fig. 1 shows how the reductone content of the reaction mixture approaches constancy.

One can see that the reducing power is almost constant between 12 and 15 minutes of heating. Longer heating always lowered the reductone content of the mixtures, probably due to further decomposition of the reductone by the alkali.

To obtain the value for the ascorbic acid equivalent of the above reductone solution, a number of 5 cc. samples taken from different 0.5 per cent glucose solutions were used. Calculated for 1 cc. of the indicator solution, they required 2.18, 2.10, 2.14, 2.06, and 2.12 (average 2.12) cc. of the heated reaction mixture. When the indicator was titrated with a freshly made 0.1 per cent ascorbic acid solution,<sup>1</sup> it required 0.55, 0.53, 0.52, and 0.55 (average 0.54) cc. of the solution per cc. of indicator. Thus, 2.12 cc. of the reaction mixture and 1 cc. of the indicator equaled 0.54 mg. of ascorbic acid. Consequently, *1 cc. of the heated reaction mixture equals 0.25 mg. of ascorbic acid.*

#### SUMMARY

Glucose solution heated under standard conditions develops a reproducible reducing capacity toward 2,6-dichlorophenolindophenol. Under the proposed conditions the resulting solution has a reducing power which is equal to 0.25 mg. of ascorbic acid per cc. of reaction mixture, which might be used for the standardization of indicator solutions when ascorbic acid is not available.

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<sup>1</sup> The author is indebted to The Rockefeller Institute for Medical Research for this sample of ascorbic acid.



# A CONTRIBUTION TO THE CHEMISTRY OF LACTO-BACILLUS ACIDOPHILUS

## III. THE COMPOSITION OF THE PHOSPHATIDE FRACTION\*

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(Received for publication, January 5, 1934)

In a former publication (1) we have shown that nearly one-third of the total lipids extracted from *Lactobacillus acidophilus* consists of a phosphatide which contained a lower percentage of phosphorus than the usual plant and animal phosphatides (2). Since no information has been published concerning the chemical composition of this material, we present a preliminary survey of the principal cleavage products which are formed when the phosphatide is hydrolyzed.

On acid hydrolysis the acidophilus phosphatide yields saturated and unsaturated fatty acids, glycerophosphoric acid, choline, and a high percentage of reducing sugars. The sugars consist apparently of a mixture of *d*-galactose, glucose, and fructose. The amount of choline that we isolated represents only a fraction of the total nitrogen. The nature of the balance of the nitrogenous compounds is unknown. We were unable to isolate any aminoethyl alcohol.

When the phosphatide is saponified with dilute alcoholic potassium hydroxide, the fatty acids are split off leaving an alcohol-insoluble polysaccharide which contains phosphorus in organic combination. When this substance is heated under pressure with dilute ammonium hydroxide, phosphoric acid is split off without liberating any reducing sugar. The phosphorus-free polysaccharide thus obtained can be crystallized in the form of small colorless prisms from dilute alcohol. The properties of the crystalline sub-

\* The data are taken from the dissertation submitted by J. A. Crowder to the faculty of the Graduate School, Yale University, 1933, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

stance do not correspond to those of any known polysaccharide but the hexose sugars liberated on acid hydrolysis are the same as those obtained from raffinose since they apparently consist of *d*-galactose, glucose, and fructose.

The low phosphorus and the high carbohydrate content of the acidophilus phosphatide shows a certain resemblance to the composition of the phosphatides that have been isolated from the acid-fast bacteria (3, 4) but the characteristic cleavage products of the latter, namely phthioic acid, mannose, and inosite, are absent.

#### EXPERIMENTAL

The purified phosphatide described in a preceding paper (1) was used in this investigation. The substance contained 1.42 per cent of phosphorus and 1.21 per cent of nitrogen. After a portion of the phosphatide had been hydrolyzed with dilute acid, it was found that the aqueous solution contained reducing sugars amounting to 21 per cent calculated as glucose.

*Hydrolysis of Phosphatide*—During the hydrolysis and subsequent operations air was displaced by carbon dioxide or nitrogen until the unsaturated fatty acids had been hydrogenated. Only freshly purified solvents were used.

The phosphatide, 49.79 gm., was rubbed into a suspension with 700 cc. of water that had been boiled and saturated with carbon dioxide. The mixture, after 12 cc. of concentrated sulfuric acid had been added, was refluxed until the coagulum which separated on acidification had been converted into an oily layer; the time required was 8 hours. After the mixture had cooled, the fatty acids were extracted with ether. The crude acids, obtained on evaporation of the ether, formed a dark brown solid mass which weighed 27.34 gm., equal to 54.9 per cent of the phosphatide. The aqueous solution was reserved for the determination of the water-soluble constituents.

#### *Examination of Fatty Acids*

The fatty acids were separated by means of the lead soap-ether method (5) into 9.13 gm. of solid saturated acids and 15.95 gm. of liquid acids having an iodine number of 72.5.

*Solid Saturated Acids*—The solid acids were methylated and the esters were fractionated and refractionated into four principal

fractions as described in the preceding paper (1) at a pressure of 0.005 to 0.001 mm. The properties of the esters were determined, after which the esters were saponified and the free acids were isolated and recrystallized.

*Fraction 1*—This fraction was evidently a mixture of palmitic acid and a lower acid but the amount obtained was too small for identification.

*Fraction 2*—Methyl palmitate, m.p. 28–29°,  $n_D^{55} = 1.4260$ . The free acid melted at 62–63° and showed no depression when mixed with palmitic acid.

0.7960 gm. acid required 30.95 cc. 0.1 N KOH  
 $C_{16}H_{32}O_2$ . Mol. wt. calculated, 256; found, 257

*Fraction 3*—Methyl stearate, m.p. 37–38°,  $n_D^{55} = 1.4302$ . The free acid melted at 69–70° and showed no depression when mixed with stearic acid.

0.3871 gm. acid required 13.59 cc. 0.1 N KOH  
 $C_{18}H_{36}O_2$ . Mol. wt. calculated, 284; found, 284

*Fraction 4*—This fraction was the residue from the first distillation. After saponification, the free acid was isolated and recrystallized from acetone from which it separated in small colorless plates which melted at 76–77°, solidified at 75°, and remelted at 76–77°. The melting point was not changed by six further crystallizations from acetone.

0.3925 gm. acid required 10.80 cc. 0.1 N KOH  
 $C_{24}H_{48}O_2$ . Mol. wt. calculated, 368; found, 363  
0.0136 gm. substance: 0.0156 gm.  $H_2O$  and 0.0390 gm.  $CO_2$   
 $C_{24}H_{48}O_2$ . Calculated. C 78.26, H 13.04  
Found. " 78.21, " 12.84

This acid apparently is a slightly impure tetracosanic acid.

*Liquid Acids*—The liquid unsaturated fatty acids were reduced with hydrogen in the presence of platinum oxide (6) and the reduced acids were separated by means of the lead soap-ether method. The solid reduced acid thus obtained weighed about 12 gm. The ether-soluble portion of the lead salts yielded 2.1 gm. of acids which solidified on standing at room temperature. Owing to the small amount of this fraction we were unable to determine



the nature of the acids that were present but the results indicate that the phosphatide does not contain any liquid saturated fatty acids analogous to tuberculostearic acid or phthioic acid (7).

*Solid Reduced Acids*—The solid reduced acid was crystallized three times from methyl alcohol yielding small colorless irregular plates. This fraction was apparently pure stearic acid. It melted at 69–70° and the molecular weight determined by titration was 285.

The mother liquors, on concentration, gave a crystalline acid which melted at 53–54° and which had a molecular weight of 261. This fraction was methylated and the ester was fractionated as described before. The principal fraction of the distillate weighed 1.5 gm. and melted at 27–28°,  $n_D^{25} = 1.4262$ .

The ester was saponified and the free acid, after it had been isolated and crystallized from methyl alcohol, melted at 62–63°.

0.3875 gm. acid required 14.98 cc. 0.1 N KOH

$C_{18}H_{32}O_2$ . Mol. wt. calculated, 256; found, 258

The data indicate that the reduced acids consist principally of stearic acid but a small quantity of palmitic acid was also present. The liquid acids contained therefore unsaturated acids of the  $C_{18}$  and  $C_{16}$  series. In addition there was present in the liquid acid fraction a small amount of an unidentified solid acid whose lead salt was soluble in ether.

#### *Water-Soluble Constituents*

*Isolation of Barium Glycerophosphate*—The aqueous portion of the hydrolysate was freed quantitatively of sulfuric acid with barium hydroxide. After removing the barium sulfate, the solution was concentrated under reduced pressure to a small volume and neutralized with barium hydroxide. A small amount of barium phosphate which separated was filtered off and discarded. The barium glycerophosphate contained in the filtrate was precipitated by the addition of 2 volumes of alcohol. The precipitate was filtered off and washed with 50 per cent alcohol. The filtrate was reserved for the isolation of the bases and carbohydrates.

The barium glycerophosphate was reprecipitated three times from water by the addition of alcohol and was obtained as a snow-

white amorphous powder which weighed 2.2 gm. For analysis the substance was dried to constant weight at  $110^{\circ}$  in a vacuum over phosphorus pentoxide.

0.2100 gm. substance: 0.1538 gm.  $\text{BaSO}_4$  and 0.0754 gm.  $\text{Mg}_2\text{P}_2\text{O}_7$   
 $\text{C}_2\text{H}_7\text{O}_5\text{PBa} + \frac{1}{2}\text{H}_2\text{O}$  (316.4). Calculated. P 9.79, Ba 43.42  
Found. " 10.00, " 43.10

*Isolation of Choline*—The filtrate from the barium glycerophosphate was freed quantitatively of barium with sulfuric acid and, after removing the barium sulfate, the solution was concentrated under reduced pressure to dryness. The residue was extracted several times with absolute alcohol and the insoluble portion was examined as will be described later for carbohydrates.

The alcoholic solution was concentrated and mixed with a slight excess of platinic chloride dissolved in alcohol. The choline chloroplatinate which separated was filtered off, washed with alcohol, and dried. The substance which weighed 0.87 gm. was twice recrystallized from dilute alcohol and was obtained in the form of orange colored crystals. For analysis the substance was dried at  $80^{\circ}$  *in vacuo* over phosphorus pentoxide.

0.2395 gm. substance gave on ignition 0.0747 gm. Pt  
 $(\text{C}_2\text{H}_7\text{ONCl})_2\text{PtCl}_4$  (615.94). Calculated. Pt 31.69  
Found. " 31.19

An effort was made to isolate aminoethyl alcohol from the filtrate from the choline chloroplatinate by means of picronic acid but none was found. The amount of choline chloroplatinate obtained accounts for only a small part of the total nitrogen. The nature of the balance of the nitrogenous compound is unknown.

#### *Examination of Carbohydrate Fraction*

The alcohol-insoluble substance mentioned under, "Isolation of choline," weighed about 10 gm. and contained reducing sugars. The material gave no pentose color reactions with orcinol but with phloroglucinol a red color was obtained. It did not contain mannose but gave a positive color reaction for a ketose sugar.

When 1.3 gm. of the substance were heated with phenylhydrazine hydrochloride and sodium acetate, 0.9 gm. of glucosazone was

obtained. The osazone, after it had been recrystallized from dilute alcohol, melted with decomposition at  $208^{\circ}$  and showed no depression in melting point when mixed with pure glucosazone.

The optical rotation of 0.1 gm. of the osazone dissolved in 4 cc. of pyridine and 6 cc. of methyl alcohol was  $-0.64^{\circ}$ . Glucosazone under the same conditions gave a reading of  $-0.65^{\circ}$ .

*Isolation of d-Galactose*—A small amount of colorless crystals separated from the syrup after it had stood for several days in a moist chamber and in the ice box. The adhering syrup was washed off with 75 per cent alcohol and the crystals were dried in a desiccator. The substance weighed 0.33 gm. and melted at  $164$ – $167^{\circ}$ .

*Rotation*—0.1372 gm. of substance dissolved in water and made up to 10 cc. gave in a 1 dm. tube a reading of  $+1.13^{\circ}$  at  $24^{\circ}$ ;  $[\alpha]_D^{24} = +82.3^{\circ}$ .

The  $\alpha$ -methylphenylhydrazone was prepared in the usual manner, 0.13 gm. of the crystalline substance yielding 0.16 gm. of the hydrazone. The hydrazone was recrystallized from 75 per cent alcohol and from a mixture of alcohol, pyridine, and water. The colorless prismatic needles melted with decomposition at  $192^{\circ}$  and showed no depression when mixed with *d*-galactose- $\alpha$ -methylphenylhydrazone.

The properties of the crystalline sugar and of the hydrazone indicate that the substance was *d*-galactose. The presence of *d*-galactose in the syrup was confirmed by the formation of mucic acid. From 1 gm. of the syrup, after oxidation with nitric acid, we obtained 0.104 gm. of crystalline mucic acid, which melted at  $212$ – $213^{\circ}$ .

*Alkaline Saponification of Phosphatide*—It has been found in previous investigations in this laboratory when the wax (8) and phosphatide (4, 9) fractions from acid-fast bacteria, which contain a high percentage of carbohydrate, are saponified with alcoholic potassium hydroxide, that the fatty acids are split off while the polysaccharide remains in the alcoholic solution as an insoluble mass. A similar saponification was carried out with the *acidophilus* phosphatide.

For the preparation of the polysaccharide 25 gm. of the phosphatide were refluxed for 7 hours with 1 liter of alcohol containing 1 per cent of potassium hydroxide. The clear solution was decanted

from a sticky mass, which adhered to the bottom of the flask, and the latter was rinsed with hot alcohol. The alcoholic solution was not further investigated.

*Isolation of Polysaccharide*—The alcohol-insoluble substance was dissolved in 25 cc. of water and, after the solution had been acidified with acetic acid, a slight excess of neutral lead acetate was added. The precipitate which separated was filtered off, washed with water, and discarded. The polysaccharide contained in the filtrate was isolated in the usual manner by means of basic lead acetate and ammonia; the lead compound being decomposed with hydrogen sulfide and the filtrate evaporated to a thick syrup. The syrup was triturated with absolute alcohol and yielded 4.2 gm. of a nearly white amorphous powder. The substance, which was strongly acid in reaction, contained phosphorus but gave no reduction with Fehling's solution until after it had been boiled for several minutes with dilute acid.

Some of the substance was dissolved in water, neutralized with barium hydroxide, and the solution was precipitated by adding alcohol. The amorphous barium salt, after it had been filtered off, washed with alcohol, and dried, was found to contain 31.37 per cent of barium and 6.6 per cent of phosphorus. Accordingly the barium-free substance would contain 9.56 per cent of phosphorus.

*Dephosphorylation of Polysaccharide*—The phosphorus-containing substance, 2 gm., was heated in a sealed tube with 10 cc. of 10 per cent ammonia to 150–155° for 6 hours. The solution was then concentrated and a slight excess of barium hydroxide was added. The barium phosphate which separated was filtered off and discarded. The filtrate was freed of barium quantitatively with sulfuric acid and concentrated to a syrup. The syrup was dissolved in 15 cc. of water; an equal volume of alcohol was added, and the solution was treated with norit, and filtered. After more alcohol had been added to the filtrate, there separated on standing and scratching 0.3 gm. of small colorless prismatic crystals. The substance had no sharp melting point; heated in a capillary tube it fused between 160–170°, effervesced slightly at about 188°, and showed no further change on heating to 250°. On drying in a vacuum over dehydrite at 78° the loss in weight was 3.75 per cent.

When the substance was hydrolyzed by refluxing with 5 per cent sulfuric acid, the maximum reduction, determined by the

Hagedorn-Jensen method (10), was attained in 2 hours and amounted to 90 per cent calculated as glucose.

**Rotation**—The substance showed mutarotation. The immediate specific rotation of the dried substance was  $+69^\circ$ , after 15 minutes the rotation was  $+60^\circ$ , and after 40 minutes a constant value of  $+72^\circ$  was obtained.

The properties of this substance do not agree with those of any known polysaccharide. The barium and phosphorus content of the amorphous barium salt is in approximate agreement with the calculated composition of a diphosphoric acid ester of a trisaccharide and the loss in weight on drying the crystalline polysaccharide corresponds to 1 molecule of water of crystallization of a trisaccharide.

One of the constituent simple sugars formed on hydrolyzing the phosphatide has been identified as *d*-galactose. The large yield of glucosazone as well as the ketose color reaction would indicate that both glucose and fructose were present. The nature of this interesting polysaccharide cannot be determined until larger quantities of the phosphatide have been prepared. From the present available data the substance appears to be an isomer of raffinose.

#### SUMMARY

The phosphatide isolated from the *Lactobacillus acidophilus* differs in composition from the usual plant and animal phosphatides.

The substance yields on hydrolysis about 55 per cent of fatty acids, the other constituents are soluble in water and consist of glycerophosphoric acid, choline, and over 20 per cent of a polysaccharide.

The fatty acids consist of saturated and unsaturated acids. The saturated acids contain palmitic acid, stearic acid, and a higher acid, probably a tetracosanic acid together with a trace of an acid lower than palmitic acid. The liquid acid fraction contains unsaturated  $C_{16}$  and  $C_{18}$  acids which on hydrogenation are converted into palmitic and stearic acids. The reduced acids also contained a small amount of an acid giving an ether-soluble lead salt but this acid could not be identified.

The polysaccharide component of the phosphatide, on acid

hydrolysis, is converted into reducing sugars consisting of *d*-galactose and apparently glucose and fructose. When the phosphatide was saponified with dilute alcoholic potassium hydroxide, a polysaccharide fraction was isolated which contained about 9.5 per cent of phosphorus. The phosphorus was split off on heating under pressure with dilute ammonia and the phosphorus-free polysaccharide was obtained in crystalline form. The substance did not reduce Fehling's solution, melted not sharply between 160–170°, and showed mutarotation; the final value was +72°.

We desire to express our thanks to the Mulford Biological Laboratories, Sharp and Dohme, for supplying large quantities of *Lactobacillus acidophilus*.

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# **A COMPARISON OF THE EFFECTS OF ADMINISTRATION OF IODIDE AND DIIODOTYROSINE ON THE IODINE AND THYROXINE CONTENT OF THE THYROID**

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In view of the hypothesis (1) that diiodotyrosine may be a step in the biosynthesis of thyroxine, as well as the recent clinical interest in its use in the preoperative treatment of hyperthyroidism (for literature see Gutman, Sloan, Gutman, and Palmer (2)) an attempt has been made to ascertain whether iodine is converted into thyroxine more readily when administered as diiodotyrosine than when given in the form of iodide.

Young guinea pigs from the stock colony ranging in weight from 200 to 300 gm. received five daily intraperitoneal injections of 2 cc. of a highly potent thyroid-stimulating fraction of beef anterior pituitary. (The writer here expresses his thanks to Dr. Karl Meyer for permission to use his unpublished method for preparing this fraction.) At the end of this time the pituitary injections were stopped, and eight animals taken at random were sacrificed (Group A). Five others (Group B) were left to recover spontaneously with no treatment other than ordinary maintenance. The remaining animals were divided into two groups, one group (Group C) receiving daily intraperitoneal injections of 1 mg. per animal of iodine in the form of KI, the other group (Group D) receiving similar injections of 1 mg. of iodine as diiodotyrosine. After three such injections of the iodine compounds seven animals of each group (Groups C and D) were sacrificed. The others received two more, or a total of five daily injections and they, together with the animals of Group B, were killed on the 6th day after the last pituitary injection.

The thyroids of all animals were removed soon after death,



freed from extraneous tissue, and dried to constant weight at 70°. A sample (usually 4 or 5 mg.) of each gland was taken for the determination of total iodine as previously described (3) except that the solution obtained on washing out the combustion tube was boiled down from about 40 cc. to about 10 or 12 cc. before acidifying and adding the bromine. This avoids the prolonged boiling of the acid iodate solution which according to Goldberg (4) gives somewhat low results. The remainder of the gland was analyzed for thyroxine by a microadaptation of the method of Leland and Foster (5) as follows: The dried gland is weighed into a short test-tube which is constricted near the bottom to form a bulb of about 2 cc. capacity; the lumen at the constriction is about 3 or 4 mm. The mouth of the tube is constricted to about 5 mm. and fitted with a tight rubber stopper. A glass bead is introduced and 2 N NaOH is added nearly to fill the bulb to the constriction. The tube is fitted with a small reflux condenser and suspended in 2 N H<sub>2</sub>SO<sub>4</sub> which is boiled under a reflux. The tube is shaken frequently until the thyroid material is dissolved in the alkali. Hydrolysis is continued for 18 hours. The tube is then cooled and the thyroxine extracted by two shakings with butyl alcohol. The level of the aqueous layer is adjusted so that the interface comes in the constricted part of the tube and the supernatant butyl alcohol layer can be quite sharply removed into a second similar tube by means of suction through a microsiphon. Gentle centrifuging causes the layers to separate sharply and rapidly. The two butyl alcohol extracts are combined in the second tube and washed by shaking with one-half their volume of 2 N NaOH. After centrifuging, the BuOH layer is blown off into a platinum boat (size 5 × 1 × 1 cm.) by means of the microsiphon tube, and the NaOH extracted with one-half its volume of BuOH. The BuOH is evaporated from the boat in a gentle air current in a steam-jacketed tube. The residue in the boat is then burned in the same manner as for total iodine, except that the large boat requires that the end of the combustion tube through which the boat is introduced be wider than the usual Pregl tubes. The method is tedious and exacting but numerous comparative analyses have shown that it gives the same results as the original method of Leland and Foster, when 2 γ or more of thyroxine iodine are present in the sample.

TABLE I

*Effect of Anterior Pituitary Injections Followed by Iodine Therapy on Iodine and Thyroxine Content of Thyroid*

Group and animal No.	Dry weight of whole gland	Whole gland		Remarks
		Total iodine	Thyroxine iodine	
	mg.	γ	γ	
A-1	15.4	1	0	Injected 5 days with thyroid-stimulating fraction of anterior pituitary; killed on 6th day
A-2	26.4	1	0	
A-3	20.0	2	0	
A-4	27.8	2	0	
A-5	21.4	Trace	0	
A-6	25.0	1	0	
A-7	18.6	Trace	0	
A-8	16.8	2	0	
B-1	22.1	6	Trace	Spontaneous recovery; killed 6 days after stopping pituitary injections
B-2	17.4	5	0	
B-3	18.5	5	Trace	
B-4	22.0	6	"	
B-5	19.4	7	1	
C-1	23.4	13	2	3 injections of 1 mg. iodine as KI; killed 4 days after stopping pituitary injections
C-2	18.8	14	2	
C-3	24.2	11	1	
C-4	21.3	19	2	
C-5	20.2	13	2	
C-6	24.5	16	3	
C-7	18.8	15	2	
C-8	25.5	19	4	
C-9	14.9	17	3	5 injections of 1 mg. iodine as KI; killed 6 days after stopping pituitary injections
C-10	17.0	21	4	
C-11	19.7	26	4	
C-12	23.1	27	4	
C-13	20.0	18	3	3 injections of 1 mg. iodine as diiodo-tyrosine; killed 4 days after stopping pituitary injections
D-1	14.6	16	2	
D-2	20.3	11	1	
D-3	12.2	17	3	
D-4	24.3	15	3	
D-5	19.5	17	2	
D-6	24.3	19	2	
D-7	21.2	14	2	
D-8	16.2	18	4	5 injections of 1 mg. iodine as diiodo-tyrosine; killed 6 days after stopping pituitary injections
D-9	20.9	26	4	
D-10	18.6	28	4	
D-11	23.3	25	4	
D-12	26.4	19	3	
D-13	19.8	26	4	

The results of the experiments are given in Table I. The figures in the third and fourth columns have been rounded off to the nearest whole number. As has been shown many times before, intensive treatment with potent pituitary fractions will cause almost complete discharge of iodine compounds from the thyroid (Group A). Thyroids of animals so treated and then allowed to recover spontaneously for 6 days are found to have regained a small amount of iodine (Group B). There appears to be no difference in the rates of return of total iodine or thyroxine whether the animals are treated with KI or with diiodotyrosine (Groups C and D).

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## FURTHER STUDIES UPON THE PURIFICATION AND PROPERTIES OF MALT AMYLASE

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Reports by Lüers and Sellner (1) upon the purification of malt amylase as well as our own experience (2) in the purification of pancreatic amylase led us to continued efforts to increase the activity and presumably the purity of preparations of malt amylase. These have resulted in the development of a new method by which preparations of much higher saccharogenic activity than any previously reported for this amylase may be consistently obtained. Additional insight into the properties of the enzyme has also resulted from this work.

The method of obtaining these highly active preparations consists of fractional precipitation of extracts of barley malt<sup>1</sup> by ammonium sulfate, solution of the most highly active fraction, dialysis to remove sulfate, repeated fractionation by ammonium sulfate with solution and dialysis of the best fraction in each case until no further increase in the activity per mg. of solid in the dialyzed solution occurs, concentration of the final dialyzed solution, fractional precipitation by alcohol, and finally precipitation by alcohol and ether.

A typical product of such a procedure formed approximately 10,000 times its weight of maltose from 2 per cent starch in 30 minutes at 40° (3) at a dilution of 1:9,000,000. The activities of these preparations on the scale of Sherman, Kendall, and Clark (4) are 3800 to 4500. Thus, they show as high sugar-forming activity as the most active preparations of pancreatic amylase so far obtained.

<sup>1</sup> Barley malt of high diastatic activity was kindly supplied by Messrs. A. Schwill and Company through the courtesy of Mr. Robert Schwartz and Mr. Oscar Ruh.

When freshly prepared, the material is readily soluble in water but becomes less soluble upon drying. It contains approximately 16 per cent nitrogen and gives the usual protein color reactions. Even in the relatively concentrated dialyzed solutions before the fractionation with alcohol, the Molisch test was negative. As purification is increased, the stability in solution is markedly decreased although to a much less extent than is observed with solutions of pancreatic amylase (5). The different conditions for optimal activity as well as other differences in properties show conclusively that malt and pancreatic amylases are different substances although of similarly high saccharogenic activity, and of protein nature.

Some of the results obtained in developing the method, and observations made upon factors which influence the results may be of interest to other investigators.

*Influence of Temperature*—In order to obtain highly active preparations it is essential that the solutions be cold. This is emphasized by Lüers and Sellner (1) and has repeatedly been found to be true in our work. For this reason, solutions were kept at all times either in a refrigerator or in ice baths. We were fortunate in having available a mechanically cooled centrifuge<sup>2</sup> which was regulated to approximately 0° so that even during the centrifuging the solutions remained cold.

*Dialysis*—Dialysis plays an important part in the purification. While this may be the source of large losses of enzyme (6) and of irregular results, it is a most useful step if suitably carried out. Many measurements of the activity of solutions of malt amylase before and after dialysis have shown that very little loss of this enzyme need occur during dialysis provided membranes free from mechanical defects are used, the temperature is maintained at approximately 0°, and the time of dialysis is kept as short as possible.

We have found Thoms' (7) dialyzers very helpful when sufficiently large volumes of solution are available for dialysis. The membranes were prepared as described by White (8) and soaked in 95 per cent alcohol for 24 hours to increase their permeability (9).

For small volumes of solution, nitrocellulose membranes which

<sup>2</sup> Manufactured by the International Equipment Company, Boston.

may be purchased<sup>3</sup> in the form of tubing of different diameters and which may be cut and tied to any desired lengths have been found very satisfactory. By placing several such "bags" in a large volume of water in the refrigerator and changing the water frequently the dialysis can be made to proceed rapidly.

*Precipitation by Ammonium Sulfate*—A systematic study was made of the use of ammonium sulfate for the separation of active from inert material in solutions of malt amylase. Fractional precipitation is more efficient than precipitation or reprecipitation with a single concentration of this salt and repeated fractionation with this reagent increases its efficiency. Typical data are given in Tables I and II.

By treating aliquot portions of a malt extract<sup>4</sup> each with a different weight of solid ammonium sulfate, including 10, 20, 30, 35, 40, 45, 50, 60, and 70 gm. per 100 cc., precipitates of different volumes and amylase activities were obtained. The greatest recovery of activity and the highest activity per volume of precipitate were found in the precipitates produced by the use of 20, 30, and 35 gm. of ammonium sulfate per 100 cc. of extract.

Fractional precipitation of malt extracts with use, consecutively, of the weights of ammonium sulfate given above, but with the removal of each precipitate as it formed, resulted in increased activity of the most active fractions which again followed the use of 20 to 35 gm. of the salt per 100 cc. of the extracts.

In order to make possible quantitative comparisons of the activities of the different fractions, each precipitate was dissolved in water and dialyzed against cold distilled water until free from sulfate. Activity and total solids were then determined in the dialyzed solutions and activity per mg. of total solid calculated. The results were also calculated to activities on the scale of Sherman, Kendall, and Clark (4). Thus expressed, the original malt extract, in a typical case, had a power of 45 and the dialyzed solutions had powers of 80, 760, 954, and 103, respectively, after the fractional precipitation with 0 to 10, 10 to 20, 20 to 35, and 35 to 40 gm. of ammonium sulfate per 100 cc. of original extract.

<sup>3</sup> Visking Corporation, Chicago.

<sup>4</sup> The extracts were prepared in all cases by mixing finely ground barley malt with 2.5 times its weight of cold distilled water, letting it stand with occasional shaking in the refrigerator for 2 hours, and centrifuging.

The solution remaining after the removal of the 35 to 40 gm. fraction had no measurable activity. Closer fractionation did not improve the results.

The power of the most active dialyzed solutions thus obtained and of the products resulting from them by subsequent alcohol precipitation may be further increased by repeating the fractional precipitation with ammonium sulfate, solution, and dialysis, several times.

In attempts to obtain as highly active final products as possible, the fractional precipitation with ammonium sulfate and dialysis are repeated until fractionation of inert material from the amylase no longer occurs and the activity of the resulting dialyzed solution fails to increase. The number of fractionations necessary to reach this point may differ with different solutions.

*Fractional Precipitation with Alcohol*—After being dialyzed free from sulfate and tested for activity, the solutions resulting from the treatment with ammonium sulfate were fractionally precipitated with alcohol. Even with such solutions, of an already relatively high degree of purification, a several-fold increase in activity is thus attained. In one case, a solution with a power of 1950 yielded a precipitate with a power of 4500 when fractionally precipitated with alcohol. This increase in saccharogenic activity per mg. of solid was accompanied by a correspondingly large increase in activity per mg. of nitrogen showing the removal of a relatively large amount of inactive nitrogenous material which had been carried along with the enzyme in the previous repeated fractionations with ammonium sulfate.

The data assembled in Tables I and II confirm previous findings (10) that fractionation with alcohol is an efficient method for the separation of inert material from malt amylase either in crude extracts or in partially purified solutions, and show that, in general, the more highly purified the solution subjected to the alcohol treatment the higher will be the saccharogenic activity of the resulting products. They also show that repeated fractionation with ammonium sulfate as well as fractionation with alcohol is necessary to remove much of the inactive material which accompanies this enzyme in extracts of barley malt.

In the fractionation with alcohol of solutions of malt amylase of widely different degrees of purity, the precipitates produced by

TABLE I

*Influence of Precipitation and Fractional Precipitation with Ammonium Sulfate upon Purification of Malt Amylase from Extracts of Barley Malt*

Experiment No.	Treatment with ammonium sulfate	Solution after dialysis	Fractional precipitation with alcohol			
			0-50 per cent alcohol	50-60 per cent alcohol	60-65 per cent alcohol	65 per cent alcohol + ether
	<i>gm. per 100 cc.</i>	<i>power</i>	<i>power</i>	<i>power</i>	<i>power</i>	<i>power</i>
1	None, malt extract	46*	80	562	142	Negligible
	0-45	744	133	284	941	1328
2	None, malt extract	46*				
	0-10	80	70		121	175
	10-20	760	500	725	951	477
	20-35	954	432	715	2680	2307
	35-40	103	32	106	326	Negligible
	Remaining solution	Negligible				

\* Not dialyzed.

TABLE II

*Influence of Repeated Fractional Precipitation by Ammonium Sulfate upon Purification of Malt Amylase*

Treatment with ammonium sulfate		Solution after dialysis	Fractional precipitation with alcohol			
			0-50 per cent alcohol	50-60 per cent alcohol	60-65 per cent alcohol	65 per cent alcohol + ether
	<i>gm. per 100 cc.</i>	<i>power</i>	<i>power</i>	<i>power</i>	<i>power</i>	<i>power</i>
1.	None, malt extract.....	54*	83	586	270	82
	20-35 gm. fraction†.....	854		1363	2300	2958
	Repeated, 20-35 gm. fraction.....	1157	355	1112	2172	2814
	"    20-35    "    "    .....	1756	387	1363	2641	3172
	"    0-35    "    "    †.....	1513		2386	3978	2000
2.	None, malt extract.....	45*				
	20-35 gm. fraction†.....	923				
	Repeated, 20-35 gm. fraction.....	1320				
	"    20-35    "    "    .....	1499				
	"    20-35    "    "    .....	1644				
	"    20-35    "    "    .....	2110				
	"    0-35    "    "    †.....	1951	1516	2750	4500	2441

\* Not dialyzed.

† In each case the precipitates obtained by the use of 0 to 10 and then 10 to 20 gm. of ammonium sulfate per 100 cc. were discarded. Those formed by the use of 20 to 35 gm. per 100 cc. were dissolved in as little water as possible and dialyzed free from sulfate.

‡ No precipitate formed upon the addition of 20 gm. of the salt per 100 cc. of dialyzed solution.



bringing the solutions to 50 and then to 60 per cent alcohol by volume were both invariably of low saccharogenic activity. More highly active final products resulted, however, if each of these fractions was precipitated and removed separately than if one 60 per cent alcohol fraction was obtained and discarded. After the removal of these precipitates, the solutions were brought to 65 per cent alcohol and after being centrifuged were treated with ether equal in volume to that of the original enzyme solution. The highest activity per mg. of solid may be found in the precipitate produced by the 65 per cent alcohol fraction, in the alcohol plus ether fraction, or in both. Precipitates produced by fractions of alcohol higher than 65 per cent were invariably low in saccharogenic activity.

While fractional precipitation with alcohol is an efficient means for the purification of malt amylase, any prolonged contact of this enzyme with alcohol causes rapid loss of activity which becomes more noticeable as the purification increases. If the precipitates produced by the alcohol and ether are immediately redissolved in water and activity and total solids determined, higher powers will usually be obtained than if the precipitate is allowed to dry on a watch crystal over sulfuric acid in a dessicator kept in the refrigerator. It is, therefore, preferable, when feasible, to study this enzyme in freshly prepared solutions of its freshly precipitated undried preparations. The decrease in power upon drying, however, is variable and may be reduced by removing the supernatant alcohol as completely as possible from the precipitates before drying. Touching the precipitates on the watch crystal with the torn edges of filter paper to remove alcohol is helpful in this connection. The products are typically protein and it is probable that the alcohol causes a denaturation of the protein with resulting loss of activity.

*Influence of Concentration of Solutions of Malt Amylase upon Activity of Final Products*—The activity of the product obtained by fractional precipitation by alcohol depends not only upon the degree of purification or activity per mg. of solid of the solution used for the fractionation as shown in Tables I and II but also upon its concentration or activity per volume of the solution. Solutions of malt amylase of approximately the same activity per mg. of solid will yield, upon alcohol fractionation, products of

widely different activities depending upon their concentrations. This is shown by the data collected in Table III in which the data for the same solutions at different concentrations are compared.

*Concentration of Solutions of Malt Amylase*—As the activities of the final products are thus markedly influenced by the concentration of the solution used for the fractional precipitation by

TABLE III  
*Influence of Concentration of Solutions of Partially Purified Malt Amylase upon Activities of Products Obtained by Fractional Precipitation by Alcohol*

Solutions			Products of fractional precipitation			
Experiment No.	Treatment of solution*		0-50 per cent alcohol	50-60 per cent alcohol	60-65 per cent alcohol	65 per cent alcohol + ether
		<i>power</i>	<i>power</i>	<i>power</i>	<i>power</i>	<i>power</i>
1	Dialyzed solution	867	343	863	1188	2310
	Concentrated 50 per cent†	936	381	990	2300	2710
	Diluted 50 per cent	867	164	450	500	1897
2	Dialyzed solution	1368	364	820	1237	2460
	Concentrated 50 per cent†	930	403	2402	2648	1856
3	Dialyzed solution	1191	390	1105	1470	1880
	Concentrated 75 per cent†	1143	233	1553	2907	2100
4	Dialyzed solution	854		1363	2300	2958
	Concentrated 25 per cent†	925	381	708	2963	2140
	" 50 " " †	893	544	1452	3173	1582
	" 75 " " †	954	673	1790	3202	1772

\* Each of these solutions was obtained by fractional precipitation of malt extract by ammonium sulfate, solution of most active precipitate, and dialysis until free from sulfate.

† Concentrated at low temperature in nitrocellulose bags as explained in text.

alcohol, the dialyses were arranged to take place with as little increase in volume of the enzyme solution as possible.

The concentration of the dialyzed solution could be further increased by using, for the dialysis, saturated solutions or even suspensions instead of solutions of the precipitates produced by ammonium sulfate. The volume of water was just sufficient to transfer the suspended precipitates to the dialyzing membranes. As the sulfate was removed by dialysis the precipitates continued

to dissolve. After such treatment the fractional precipitation by alcohol and ether gave products of higher activities than had previously been obtained.

Lutz (11) has found it possible to concentrate solutions of invertase by evaporation brought about by fanning in air the nitro-cellulose bags which contain the solutions. This is a suitable way in which to concentrate solutions of malt amylase also. The rapid evaporation keeps the solutions cool and usually little if any loss in activity occurred when the dialyzed solutions were thus concentrated 50 to 75 per cent. This is shown by the powers given in Table III for solutions before and after concentration. In a typical experiment a solution which was concentrated in this way to approximately one-half its volume caused the formation of 344 mg. of maltose per 0.1 cc. before and of 685 mg. of maltose per 0.1 cc. after the concentration.

Partial freezing has been suggested as a method for the concentration of enzyme solutions. It was found in this investigation that relatively dilute solutions of malt amylase may readily be concentrated in this way, the enzyme being concentrated in the unfrozen portion of the solutions. With dilute solutions very little loss of enzyme occurred either by removal with the ice or through inactivation. As the freezing of a solution was repeated and the solution became more concentrated, however, marked inactivation of the enzyme occurred. This is probably due to a denaturation of the protein such as occurs with other typical proteins under similar conditions.

*Adsorption by Alumina Gel*—Adsorption of the enzyme from its solutions by alumina gel (1) with its subsequent elution (12) from the gel was introduced at different stages of the purification process. These steps were found to be relatively inefficient in the purification of this enzyme when compared with the precipitation by ammonium sulfate and alcohol described here, and their use in conjunction with the precipitation procedures did not increase the activity of the final products.

#### SUMMARY

A method for the purification of malt amylase which yields products of much higher saccharogenic activity than any previously reported for this enzyme has been developed. It is described briefly and discussed.

The material thus purified contains about 16 per cent nitrogen, shows all the ordinary protein color reactions, and also behaves like typical protein in respect to precipitation and denaturation. The denaturation of the protein and the loss of enzymic activity coincide.

In respect to the relative prominence of its starch-splitting and sugar-forming activities, the material purified by the method here described appears to correspond to the  $\beta$ -amylase discussed by Kuhn (13).

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## THE SYNTHESIS OF SERINE\*

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The methods described in the literature for the synthesis of serine are not well adapted to the preparation of this amino acid in the quantity and with the economy required in many biochemical investigations. Fischer and Leuchs' (1) synthesis from ammonia, hydrocyanic acid, and glycolaldehyde appears to be impracticable because this aldehyde is conveniently prepared only in dilute aqueous solution and is known to polymerize readily. The method of Erlenmeyer and Erlenmeyer and Stoop (2, 3), based on the reaction of formic ester with sodium ethylate and hippuric acid ester, is of doubtful value because of the numerous steps and the tedious manipulations. A synthesis, proposed in 1930 by Mitra (4), appears to be promising since yields up to 28 per cent were reported. However, only 2 gm. of serine were prepared by this method. The procedure of Leuchs and Geiger (5) has been more widely used (6-8) than other methods, yet its usefulness is diminished by the experimental difficulties encountered in the preparation of chloroacetal (9-11) and the intermediate substances, ethoxyacetal (5, 9, 12) and ethoxyacetaldehyde (5, 13, 14).

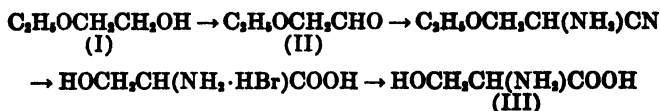
Two unsuccessful attempts to prepare serine have been reported. Erlenmeyer and Erlenmeyer and Früstück (15, 16) prepared phenylserine by condensing benzaldehyde with glycine, but the comparable reaction between formaldehyde and glycine gave negative results. Although a crystalline amino derivative was

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produced when ammonia was allowed to react with the chlorohydroxy acid, formed from hypochlorous and acrylic acids, the investigations of Melikoff (17) and Erlenmeyer (18) indicated that this product was isoserine. In our experiments with  $\alpha$ -chloro- $\beta$ -hydroxypropionic acid it was assumed that the entering amino group could be directed to the  $\alpha$  position if first the hydroxy radical were stabilized by the formation of an immobile derivative. However, the results with the acetyl, benzoyl, and *p*-nitrobenzoyl esters of this acid were unsatisfactory. Our further attempts to synthesize serine through the intermediate  $\beta$ -methoxypropionic acid were likewise unsuccessful.

The present synthesis of serine is based on the following reactions.



Prior to the adoption of the conventional methods described in the experimental part for the oxidation of ethylene glycol monoethyl ether (I) several catalytic procedures were investigated. In these trials the vaporized glycol ether was exposed to the action of atmospheric oxygen in the presence of the catalysts cupric oxide and copper (19), copper-chromium oxide (20), or silver oxide at temperatures between 300–400°. Air was passed through the system for about 1 hour at the rate of 1 liter per minute as measured by a calibrated flowmeter. It was apparent that oxidation had occurred, since the catalysts were reduced and the distillate gave a strong aldehyde odor and Schiff's test. However, it was later found that only a small percentage of aldehyde had been formed under the observed experimental conditions.

Pure ethoxyacetaldehyde (II), b.p. 71–73°, was isolated by Klüger (13) and Eissler and Pollak (14) but the methods employed by these authors are not suited to the preparation of the aldehyde in the quantity desired in our work. It was finally considered most feasible to use a water solution of the aldehyde, since fractionation by ordinary methods was found to be impracticable, if not impossible. The fraction, b.p. 70–95°, was used for the further reactions, while the fraction, b.p. 84–95°, was uti-

lized by Leuchs and Geiger (5). Additional experiments were performed with ethylene glycol monomethyl ether on the assumption that the aldehyde resulting from the oxidation might be readily separated from its water solution by fractional distillation. It was found, however, that the fractionation of methoxyacetaldehyde was more difficult than with ethoxyacetaldehyde.

The iodometric-bisulfite method of Donnally (21) gave satisfactory results in the determination of the ethoxyacetaldehyde content of the aqueous fraction. The attempts to obtain a quantitative measure of the aldehyde by means of the 2,4-dinitrophenyl-



FIG. 1. Photomicrograph of *dl*-serine.  $\times 80$

hydrazone derivative or by volumetric and gravimetric procedures with cupric ion as an oxidizing agent were unsuccessful.

In general the method outlined by Leuchs and Geiger (5) for the conversion of ethoxyacetaldehyde (II) to serine (III) was followed in our experiments. It seems probable, however, that the suggestions of Cocker and Lapworth (22) for the elimination of ammonia and ammonium salts and those of Orten and Hill (23) for the crystallization of the amino acid could be employed to advantage here. The modified Strecker technique has been utilized for the synthesis of glycine and alanine but it is considered to be less satisfactory for most aldehydes (22) than the original ammonia-hydrocyanic acid method.

It seems probable that the yield of serine can be increased some-



what, since 14.7 gm. (61.3 per cent of theory) of crude product were obtained in a preliminary experiment. The Van Slyke amino nitrogen values for serine were found to be 4.5 per cent higher than the theoretical. This abnormality is in agreement with that reported by other workers. The photomicrograph of serine (see Fig. 1) is similar to that given by Keenan (24).

#### EXPERIMENTAL

*Anhydrous Hydrocyanic Acid*—Anhydrous hydrocyanic acid was prepared by a method essentially like that described by Ziegler (25).

*Ethoxyacetaldehyde (II)*—A 2 liter three-necked flask was fitted with a mechanical stirrer, a dropping funnel, and a short still head. The latter was equipped with a thermometer and connected to a long water-cooled condenser placed for downward distillation. 270 gm. (3 moles) of ethylene glycol monoethyl ether (I), b.p. 133–133.4°, and 100 cc. of water were placed in the flask. A mixture composed of 330 gm. (1 mole) of technical sodium dichromate ( $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ ), 400 cc. of water, and 240 cc. of concentrated sulfuric acid was placed in the separatory funnel.

The mixture in the flask was stirred rapidly and heated to boiling. The oxidizing mixture was added to the boiling liquid at the rate of 3 or 4 drops per second and the heat adjusted so that the distillate collected at about the same rate. About 45 minutes were required to complete the reaction, during which 500 cc. of distillate, b.p. 99–101°, collected in the receiver. Upon fractionation of the distillate with a 24 inch column, filled with glass beads and fitted with a partial condenser, 84 gm. of aqueous aldehyde solution, b.p. 70–95°, were obtained. The largest part of this fraction came over between 90–95°.

The ethoxyacetaldehyde content of this aqueous fraction was approximately 46 per cent, as shown by an analysis for aldehyde by the method of Donnally (21). Hence the yield was 14.7 per cent of the theory.

*Serine (III)*—167 gm. (0.87 mole of pure aldehyde) of 46 per cent aqueous ethoxyacetaldehyde solution and 250 cc. of 7 N ammoniacal methyl alcohol (1.75 moles) were placed in a 500 cc. flask. When these solutions were mixed, much heat was produced and the alcohol started to distil. The mixture was allowed

to stand for about 12 hours, after which 40 cc. (1 mole) of liquid anhydrous hydrocyanic acid were added. The resulting warm solution was placed in the dark to minimize the polymerization of the hydrocyanic acid to azulmic acid compounds (26). The solution was permitted to remain in the dark for about 3 days. During this time the initial yellow color changed to a deep red but the solution remained transparent. It is probable that the total time for these reactions may be shortened to a few hours, according to the experience of Cocker and Lapworth (22) with other aldehydes.

The red-colored reaction mixture was placed in a 1 liter distilling flask fitted with a rubber stopper and a dropping funnel. The side arm of the flask was connected to a long water-cooled condenser which led to a trap containing 6 N sodium hydroxide for the absorption of excess hydrocyanic acid. 400 cc. of 40 per cent hydrobromic acid were added to the flask through the dropping funnel and the mixture was heated to boiling. When most of the alcohol and some of the water had distilled, an additional 100 cc. of 40 per cent hydrobromic acid were added and the distillation continued. When the distillate became turbid owing to droplets of ethyl bromide the heat was moderated and distillation continued until about 20 cc. of ethyl bromide had collected as an oily layer in the receiver. Another 100 cc. of 40 per cent hydrobromic acid were added and slow distillation continued until oily drops of ethyl bromide were no longer visible in the distillate. A total time of 6 hours was required for the hydrolysis, during which considerable solid material separated and the reaction mixture became colored a deep brown.

The residual liquid in the flask was diluted with an equal volume of distilled water, heated to boiling, treated with 30 gm. of decolorizing carbon, and filtered. The filtrate was evaporated nearly to dryness under reduced pressure to remove the excess hydrobromic acid, 1.5 liters of distilled water added to the crystal mass, and the mixture transferred to a 3 liter beaker. The mixture was stirred vigorously, heated to boiling, and powdered lead carbonate added to the neutral point. The lead bromide was filtered and thoroughly washed with several portions of distilled water. 200 gm. of lead carbonate were added to the combined filtrate and washings and the mixture was boiled with constant stirring to remove

the bulk of the ammonia. Freshly precipitated lead hydroxide, prepared from 200 gm. of lead nitrate and an excess of dilute ammonia, was added to the mixture which was boiled until the vapors contained only traces of ammonia. The insoluble lead salts were filtered and washed thoroughly with distilled water. The combined filtrate and washings were treated with hydrogen sulfide and the precipitated lead sulfide filtered and washed.

The filtrate was evaporated under reduced pressure to about 400 cc. and treated with 10 gm. of decolorizing carbon. An equal volume of 95 per cent ethyl alcohol was added to the clarified filtrate and the mixture allowed to stand overnight in the ice box. A total of 42 gm. (64 per cent of theory) of almost white serine (III) was obtained when the crystals were filtered, washed with alcohol, and dried to constant weight at 65°. Upon recrystallization from 500 cc. of 50 per cent ethyl alcohol 36 gm. (40 per cent of theory) of pure serine were obtained.

$C_2H_5O_2N$ . Calculated, N 13.33; found (Kjeldahl), 13.21, 13.22, 13.55, 13.12; (Van Slyke), 13.96, 13.95, 13.91

#### SUMMARY

A 46 per cent aqueous solution of ethoxyacetaldehyde, prepared by the oxidation of ethylene glycol monoethyl ether with sodium dichromate and sulfuric acid, has been used for the synthesis of 36 gm. of analytically pure serine. The described procedures are both convenient and inexpensive.

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## THE SEXUAL VARIATION IN CARBOHYDRATE METABOLISM

### III. THE COMPARATIVE GLYCOGEN AND FAT CONTENT OF THE LIVER AND MUSCLES OF RATS AND GUINEA PIGS\*

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Earlier papers from this laboratory have established the fact that normal women excrete much larger quantities of acetone bodies in the urine during fasting than do normal men and that similar discrepancies occur between male and female rats and guinea pigs during inanition when these animals are fed sodium acetoacetate (1, 2). Inasmuch as this variability continues for a period of at least a week, it is suggested that there must be a fundamental difference in carbohydrate metabolism between the sexes.

Although it is evident from the extended duration over which the variation in ketonuria has been observed that its explanation cannot lie alone in a larger carbohydrate reserve in the male at the beginning of the fast, yet it would seem that the level of liver glycogen might differ at any period of fasting according to the sex of the animal. Such a difference, which might be ascribable to sex, has not, however, been generally recognized. In fact, in some cases animals of either sex have been used indiscriminately for studies of glycogenesis.

Greisheimer (3) first clearly pointed out that male rats which were fed on various diets had higher liver glycogen and lower liver

\* This work was aided by Grant 289 from the Committee on Scientific Research, American Medical Association. Preliminary reports of some of these data have been published (Deuel, H. J., Jr., Samuels, L., and Gulick, M., *Proc. Soc. Exp. Biol. and Med.*, **30**, 27 (1932-33); Deuel, H. J., Jr., *J. Biol. Chem.*, **100**, xxxv (1933)).

fat than did the female animals on similar dietary régimes. An analysis of the results of Ponsford and Smedley-MacLean (4) shows like differences, although these investigators do not mention the discrepancies. Thus, the values given by these authors for liver glycogen of rats fed diets high in the salts of the following organic acids may be subdivided in each case into the following percentages according to sex: control 1.08 (0.95,<sup>1</sup> 1.10);<sup>2</sup> acetate 1.26 (2.28, 0.97); fumarate 2.13 (2.40, 1.95); malate 2.18 (2.67, 1.45); succinate 2.27 (2.80, 2.13); glucose 3.50 (—, 3.50). The liver fats show even more convincing differences as follows: control 8.15 (7.43, 9.05); acetate 5.71 (4.64, 6.24); fumarate 4.04 (3.10, 4.73); malate 4.46 (4.04, 5.09); succinate 4.41 (3.90, 4.81); glucose 5.59 (4.34, 5.73). It is obvious that in all cases but one the liver glycogen is higher in the male rats, while in every instance the average liver lipid is greater in the female animals. The differences are more pronounced if one considers only those pairs of animals which are litter mates. In making a comparison of the glyco-genetic ability of various substances, it is evident that the average level obtained depends not alone on the diet which is being fed but to a very great extent on the relative proportion of male and female animals used in making the tests.

Stöhr (5) has reported experiments on young rats after short periods of fasting which show similar differences in liver glycogen, although the levels are lower than we have found. It should be noted, however, that opposite results were obtained on groups of five old rats of each sex which were of undetermined age and which were not litter mates.

The experiments given in the present paper were undertaken to determine whether such a variation in liver glycogen could be demonstrated after various periods of fasting (up to 120 hours) following the administration of similar amounts of glucose to male and female rats. It was also desired to ascertain whether there might be such a variability in another species, such as the guinea pig. It also seemed desirable to make comparisons of the level of liver lipid in these cases.

<sup>1</sup> Only one male animal in this experiment.

<sup>2</sup> The first value in parentheses following the average of Ponsford and Smedley-MacLean is for male rats, while the second one is the average for the females.

## EXPERIMENTAL

*General Procedure*

Normal adult rats which were litter mates as far as possible, obtained from our stock colony, were used. The guinea pigs were from the same source as those employed in our earlier work (2). In order to minimize any differences in glycogen content during fasting which might be ascribable to variations in the amount of carbohydrate in the gastrointestinal tract at the start of the fast, the animals were subjected to a preliminary fast period of 48 hours, after which time 5 mg. of glucose per sq.cm. of body surface were administered by stomach tube in 50 per cent solution. In this way the carbohydrate store of the rats and guinea pigs was brought to approximately the same level at the start of the experimental (second) fast. In order to avoid any possible variations due to diurnal changes in glycogen storage as suggested by Forsgren (6) as well as by Agren, Wilander, and Jorpes (7), but denied by Higgins, Berkson, and Flock (8), we have in most cases killed our animals at uniform periods, namely from 8.30 to 10.00 a.m. Some of the guinea pigs were killed as late as 2 p.m.

During the whole of the fasting period the animals were kept in a warm room to obviate loss of glycogen by shivering. 90 mg. per kilo of amytal were given intraperitoneally as an anesthetic. Liver and muscle glycogen were determined as described previously (9).<sup>3</sup> In most cases the gastrocnemius muscle of the right

<sup>3</sup> Because of discrepancies between our results on liver glycogen and those of other observers, our detailed method of analysis is given. As soon as anesthesia was complete (3 to 5 minutes), the muscle was removed and frozen in a CO<sub>2</sub>-ether mixture. The liver was then excised as quickly as possible while the animal was still alive. The adherent blood was removed by pressing with paper towels, and the sample immediately frozen. The whole procedure from the start of the operation required slightly over 2 minutes. When fat determinations were to be made, the liver was separated into approximately equal parts and only that used for glycogen was frozen.

The samples of muscle and liver were weighed while still frozen, placed in a 200 × 25 mm. test-tube, and hot 40 per cent KOH was added (approximately twice the volume of liver or muscle sample). The tubes were immediately placed in a boiling water bath after being covered with tin-foil. They were shaken at frequent intervals until the tissues were dissolved. Digestion was continued for 3 hours. After the removal from the bath, 2



leg was used. The ether-soluble fraction of the liver was determined on ground samples of the material which had been dried in a vacuum oven at 50°. Blood sugar determinations were made by the Shaffer-Hartmann procedure.

Only female rats which showed a normal estrual cycle as demonstrated by vaginal smears were used. Surface area was calculated in most cases by the formula of Lee (10), although in a few of the earlier tests the formula of Meeh was employed. The differences are so small that these results may be compared directly with the later ones. The reasons for basing the dose of glucose on surface area rather than on body weight are discussed in Paper II (2). The recent results of MacKay and Bergman (11) published since that time seem further to justify this practice.

*Experiments on Rats*—A summary of the results on 263 rats is reported in Table I. The experiments on the male and female animals in each group for each fast period were carried out simultaneously. The determinations on Groups I and II were carried

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volumes of 95 per cent alcohol were added while the contents of the tubes were still slightly warm. This avoids difficulty in mixing, due to the separation of soaps. They were then allowed to stand overnight. On the following day they were filtered through Munktell filter paper (No. 1 F). We have found considerable loss to occur with known glycogen samples when Whatman No. 1 is used. Thus, an average of four experiments in which the latter paper was employed was 8.4 per cent lower than the mean of four analyses with Munktell paper. In one case the value with Whatman paper was 13.8 per cent less.

After washing the precipitate successively with 66, 95, and 100 per cent alcohol and ether, and then again with absolute alcohol, it was dissolved with boiling 0.5 N HCl and allowed to drain into the original tube which usually contains some of the adherent glycogen. It was hydrolyzed in a boiling water bath for 3 hours. After cooling, it was made up to an appropriate volume and the glucose determined by the Shaffer-Hartmann procedure. Glycogen is calculated from glucose by multiplying by the usual factor, 0.927. We have found that the hydrolyzed glycogen solutions can be kept without change for at least 48 hours. On the other hand, when the precipitated glycogen is allowed to stand for periods longer than 1 day at room temperature, appreciable losses were found to occur. Thus, the average purity of a glycogen sample hydrolyzed without precipitation or after precipitation and standing for 48 hours was found to be 92 per cent. The percentage of recovery had fallen after standing for 5 days to 87.4 per cent, after 8 days to 85.0 per cent, after 15 days to 75.8 per cent, and after 18 days to 77.8 per cent.

TABLE I

*Glycogen and Fat Content of Liver and Muscle Glycogen, in Per Cent, in Male and Female Rats Fasted for Various Periods*

Fast period	Group No.	No. of rats		Liver						Muscle glycogen	
				Glycogen			Fat				
		Male	Fe- male	Male	Fe- male	A:B*	Male	Fe- male	A:B*	Male	Fe- male
hrs.											
0	I, a	10	10	3.32	3.32		3.11	3.20		0.359†	0.326†
	I, b	10	10	3.77	3.74		3.01	3.49		0.427	0.402
Average.....		20	20	3.54	3.53		3.06	3.34		0.427	0.402
24	I	5	5	1.20	0.66		3.90	4.53		0.344	0.359
	II	10	10	1.54	0.93					0.394	0.404
Average...		15	15	1.43	0.70	6.40	3.90	4.53	2.52	0.369	0.383
48	I	10	9				5.05	5.27		0.331	0.354
	II	5	6	0.38	0.09					0.281	0.278
	III	19	16	0.47	0.31					0.253†	0.246†
	IV	10	10	0.39	0.21		3.19	4.62		0.239†	0.257†
Average....		44	41	0.45	0.25	6.25	4.22	4.92	2.41	0.303	0.308
72	I	10	10	0.31	0.13		4.44	4.55		0.217	0.225
	II	8	10	0.31	0.12					0.251	0.233
Average...		18	20	0.31	0.13	6.67	4.44	4.55		0.235	0.229
96	I	9	9	0.41	0.29	2.03	3.98	5.02		0.238	0.240
	II	7	7	0.31	0.49	1.18				0.288	0.242
Average. ...		16	16	0.36	0.37		3.98	5.02	2.60	0.260	0.241
120	I	10	9	0.33	0.50	2.40	3.89	5.10		0.257	0.272
	II	10	9	0.44	0.43					0.251	0.184
Average.....		20	18	0.39	0.47		3.89	5.10	2.85	0.254	0.228

\* Ratio of mean difference to probable error of mean difference. This was calculated by the following procedure.

P.e. (individual determination) = a.d.  $\times$  0.8453

" (mean) = p.e. (individual determination)/ $\sqrt{n}$

" ( " difference) =  $\sqrt{(p.e. (mean A))^2 + (p.e. (mean B))^2}$

When the ratio of mean difference to probable error of mean difference exceeds 3, the results are considered significant.

† Determinations made on all muscles of rear leg instead of gastrocnemius; not included in average.

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out in June, 1933, while those on Groups III and IV were made during February and March, 1932.

With the exception of the rats which were killed without previous fasting, each group up to that at 96 hours shows values for liver glycogen in the males which are significantly higher from the statistical standpoint. We have been unable to find any such discrepancy with the animals which were killed without a previous fasting period. There is no difference in the grand average of the 96 hour group, although one subgroup shows higher values for the males, while the other one has a similar condition for the females. In the 120 hour series, the liver glycogen appears to be highest in the females. The level of liver glycogen rises abruptly in these

TABLE II

*Liver and Muscle Glycogen and Liver Fat, in Per Cent, in Male and Female Rats Killed after 48 Hours of Fasting Following Diets High in Glucose, Galactose, and Lactose*

Prefasting diet	No. of rats		Body weight		Liver				Muscle glycogen*	
					Glycogen		Fat			
	Male	Fe- male	Male	Fe- male	Male	Fe- male	Male	Fe- male	Male	Fe- male
			gm.	gm.						
Glucose.....	2	4	237	176	0.49	0.12	4.14	5.97	0.18	0.22
Galactose.....	4	1	200	152	0.89	0.38	3.54	5.05	0.30	0.25
Lactose.....	3	3	204	170	0.55	0.22	4.02	5.56	0.22	0.26

\* Rear leg muscles.

last two periods in the female animals, while that of the male rats is only slightly changed from the level obtained after a 72 hour fast. Although the liver fat is consistently higher in the females than in the males, the averages for any single group are not statistically significant because of the small number of animals on which this value was determined.

The muscle glycogen declines with the progress of the fast but there is no appreciable difference between results for the sexes. In only two cases were significant variations noted; *i.e.*, in the 96 and 120 hour series of Group II in which the values for males were higher.

The same difference for the sexes in liver glycogen and fat is indicated in a small group of animals which were fasted for 48 hours

after having been fed on special diets high in glucose, galactose, or lactose which are described elsewhere (9). A summary of these data is given in Table II.

*Experiments on Guinea Pigs*—The technique for the experiments on guinea pigs was similar to that employed with rats. Only one period of fasting after glucose was studied, i.e. 48 hours. The three groups of experiments are summarized in Table III.

TABLE III  
*Average Percentage of Water, Glycogen, and Fat in Livers and Muscle Glycogen of Male and Female Guinea Pigs Following 48 Hour Fast*

Group No.	No. of animals		Liver						Muscle glycogen		Blood sugar	
			Water		Glycogen		Fat					
	Male	Fe-male	Male	Fe-male	Male	Fe-male	Male	Fe-male	Male	Fe-male	Male	Fe-male
I	8	11	70.7	68.8	0.21	0.20	3.48	6.44	0.50	0.55	126	142
II	10	8	72.1	69.8	0.16	0.52	3.32	5.45	0.53	0.58	124	133
III	14	14	71.4	70.4	0.18	0.56	4.92	6.68	0.34	0.41		
Average ..	32	33	71.4	69.7	0.18	0.43	4.03	6.30	0.44	0.50	125	138

#### DISCUSSION

The liver glycogen has been found to be uniformly higher in male rats than it is in the females for all periods of fasting up to 96 hours after the administration of glucose. This may be caused by a greater speed at which carbohydrate is oxidized in the female than it is in the male or to a more rapid transformation of this foodstuff into fat. Another possibility is that the formation of ketolytic material may be qualitatively or quantitatively different in the female than it is in the male.

In one group of animals killed after a 96 hour fast and in both series after a 120 hour period of inanition, there is a sharp rise in the liver glycogen in the female animals from that found after 72 hours. Since little change was observed in the male animals in these later periods from the level noted after 72 hours, the average value for the females becomes equal to or greater than that for the males. The reason for this increase in liver glycogen is problematic but it was associated in two instances with a considerable

fall in muscle glycogen in the females when compared with the corresponding group of males. It may be that this is in the nature of a premortal rise during which all possible glycogen stores are mobilized in the liver even at the expense of muscle glycogen.

When no period of fasting preceded the killing of the rats, the averages of liver glycogen in the males and females are unusually close. These results are not in harmony with those which we have interpreted from the work of Ponsford and Smedley-MacLean (4) when no period of fasting occurred prior to the death of the animals. One possible explanation for the difference is that the stock diet which we employed was particularly high in carbohydrate (9), while those of Ponsford and Smedley-MacLean were high in the salts of various organic acids. We are at present making a study of the effect of diet on the glycogen content of the livers of male and female rats without previous fasting. Another possibility is that since our animals were killed early in the morning there would be a minimum period of fasting inasmuch as the rat is an animal which eats largely at night. On the other hand, if the rats of Smedley-MacLean were killed late in the afternoon, a sufficient period of fasting might have occurred so that the sexual difference would become evident. In our experiments in which pronounced sexual variations were found 24 hours after the feeding of glucose, the fast must represent a maximum of 18 hours after the complete absorption of the monosaccharide from the gut.

The level of liver glycogen (1.43 per cent) which we found for male rats following a 24 hour fast after glucose feeding is much higher than most of the figures reported in the literature. Karozag, Macleod, and Orr (12) and Barbour, Chaikoff, Macleod, and Orr (13) give 0.16 per cent as the average value for twenty-four rats in separate sets of experiments. Catron and Lewis (14) report a mean value of 0.09 per cent for sixteen rats, while Silberman and Lewis (15) give a figure of 0.05 per cent. In none of the above investigations is the sex of the animals noted. In tests in which male rats alone were used, Lawrence and McCance (16) have obtained a mean of 0.40 per cent on eight rats, while Cori and Cori (17) report an average figure of 0.10 per cent on eight young rats (average weight 116 gm.). Eckstein (18) has recently published results in which the figures vary between the averages of 0.10 and 0.06 per cent for forty-four rats.

The higher levels which we have found for male rats fasted for 24 hours may be partially explained by the large amount of carbohydrate given to our animals prior to the beginning of the fast. The glucose which was fed probably gave the animal a much greater reserve of carbohydrate than obtained in the experiments mentioned above, especially if the stock diet had been one low in this foodstuff. However, we have never obtained an average lower than 0.31 per cent in male rats, even after a fasting period of 120 hours. In the 24 hour group it also would seem that the effect of the glucose might be counterbalanced by the lower carbohydrate store at the time when the monosaccharide was administered, owing to the preliminary 48 hour fast which we employed. Although 1.43 per cent is an average for only fifteen male animals, it should be pointed out that there are fourteen additional observations on female rats fasted twenty-four hours in which the mean figure is 0.70 per cent.

We are certain that the high levels which we have obtained are not to be attributed to improper analytical technique. We have frequently checked our recoveries on known samples of glycogen carried through the entire procedure and have always obtained satisfactory results. It is difficult to see why any errors in technique should result in a greater recovery of glycogen when all the possibilities in the removal of the liver and in the analytical procedures are for a loss to occur.

The greater uniformity between our results and those of other investigators following a 48 hour fast also supports the correctness of our analyses. After 48 hours of fasting, Greisheimer (3) reports average values of 0.513 and 0.137 per cent respectively for three male and nine female rats, while our results are 0.45 and 0.25 per cent for thirty-four male and thirty-two female rats. Cori (19) reports an average of 0.397 per cent on seven male rats, while Lawrence and McCance (16) have found one of 0.30 on a group of eight males. Barbour, Chaikoff, Macleod, and Orr (13) obtained a level of 0.32 per cent with sixty-eight rats of which the sex is not noted.

The liver lipid averages higher in the female rats than in the males but the number of determinations in each group (which is much less than the number of glycogen estimations) is too small for the differences to be considered significant. However, if one

takes an average of all the lipid values of the livers of fasting rats, irrespective of length of the fasting periods, one finds that the grand average for all males of 4.11 per cent (50 rats) is significantly lower than the mean value for females of 4.83 per cent (53 rats). In this case the ratio of mean difference to probable error of mean difference is 5.07, a value which indicates that the levels are definitely variable statistically.

In the experiments on guinea pigs in which there was only one fast period of 48 hours, opposite results were obtained for liver glycogen from those on rats after a similar length of fast. The average for the males was 0.18 per cent, while that for the females was 0.43 per cent. On the other hand, the liver fat was significantly higher in the female guinea pigs than it was in the males—a result in harmony with the experiments on rats. Thus, the average lipid content of 6.30 per cent in the females was approximately 50 per cent higher than the value of 4.03 per cent found for the males. The ratio of mean difference to probable error of mean difference in this case is 5.97.

In our studies on the sexual difference in carbohydrate metabolism the only aberrant results are those for liver glycogen in the guinea pigs. The readier development of ketonuria in women than in men, which has been supported by the recent work of Hawley, Johnson, and Murlin (20), is similar to the results on fasted male and female rats and guinea pigs which are fed sodium acetoacetate. Not only was the greater ketonuria in the female rats proved statistically, but it has been substantiated on a larger group of animals by work from this laboratory (21, 22). The variations in the liver glycogen of female rats following ovariectomy (23) give further proof of the marked effect which the ovary must exert on the level of liver glycogen.

It is generally considered that an inverse ratio exists between the level of liver fat and liver glycogen. Thus, Lusk (24) states that "in the liver there is an antagonism between glycogen deposit, which follows carbohydrate ingestion, and fat deposition." The experiments of Greisheimer (3), Ponsford and Smedley-MacLean (4), and those on rats fasted for periods up to 96 hours in the present paper (Tables I and II) do show such a relationship. If one accept such a hypothesis, then one must consider either that such a relationship does not exist in guinea pigs or that the results

on liver fat or glycogen are incorrect. It seems highly probable that the liver fat is much less rapidly altered by experimental procedures than is liver glycogen, so the liver lipid would be the more reliable index of sexual differences. Moreover, the experiments on guinea pigs were made on animals for which no reliable records of age or parentage were available. This, coupled with the fact that some glycogenolysis must have occurred before the removal of the liver (since high blood sugars obtained), would seem to be sufficient reason for discounting the results on liver glycogen in these animals. In addition, the fact that male guinea pigs oxidize a larger proportion of diacetic acid than do female animals (2) offers additional proof of the similarity in carbohydrate metabolism in these animals to that in rats.

#### SUMMARY

1. The liver glycogen was higher in fasting male rats killed 24, 48, and 72 hours after the administration of glucose than it was in females. An abrupt rise occurred in the glycogen level in the female rats in one case after 96 and in two groups after 120 hours, which brought this constituent to a higher level than in males. It is suggested that this may be in the nature of a premortal rise.

2. The liver fat was higher in the female than in the male rats during the whole period of fasting.

3. There were no differences in glycogen level between the sexes in rats killed without fasting after a high carbohydrate diet.

4. The liver glycogen was found to be higher in female than in male guinea pigs after 48 hours of fasting. Possible explanations for this discrepancy are offered. However, differences, due to sex, in liver lipid similar to those found in rats were obtained with guinea pigs.

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# THE SPECTROSCOPIC IDENTIFICATION OF PHENYL- ALANINE IN PROTEIN MATERIAL

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## PLATE 1

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A preliminary survey of the literature, in which numerous aromatic derivatives were investigated, indicated that nearly all those compounds having a light-absorbing group or negative substituent directly attached to the benzene nucleus were characterized by general absorption in the ultra-violet region; *i.e.*, had spectra consisting of no bands at all or else one or two broad diffuse bands. On the other hand, those having one or more methylene ( $\text{CH}_2$ ) groups between the nucleus and additional absorbing group had sharp absorption—three or more distinct bands. Since this was found to be true in the case of numerous hydroxyl derivatives, acids, aldehydes, and many other varied classes of compounds, we believed that it would also apply to the aromatic amino acids, and that phenylalanine would have several sharp bands, while tyrosine and tryptophane would have only one or two diffuse bands. Most of the naturally occurring aromatic compounds, especially those found in protein material, fall in the larger group of general absorption and thus should not be confused with phenylalanine.

Studies of the aromatic amino acids as reported in the literature are very unsatisfactory in that very few of the results check with each other (1-10). Therefore, we first investigated the spectra of these acids as individuals. Phenylalanine was found to have five distinct bands at 2680, 2640, 2585, 2525, and 2480 Ångström units. This was to be expected since there is one complete methylene group between the nucleus and other functional groups. However, only one previous worker, Ward (3), has reported a banded spectrum of this nature and the position of the bands as interpreted from his graph is not in complete agreement with our

results. Shibata and Asahina (6) reported two bands, 2645 and 2564 Ångström units, while other investigators found only one at 2700 or 2565 ((8, 9) respectively). The absorption of tyrosine because of its phenolic hydroxyl group, is dependent upon the pH of the solution. In alkaline solution we found two bands, at 2840 and 2760 Ångström units, which coalesce upon concentrating. These agree with the values 2800 and 2750 Ångström units at pH 12.7 reported by Stenström and Reinhard (4) who investigated the effect of varying the hydrogen ion content of the solvent. Other values reported in the literature (1-3, 6, 8, 9) vary considerably, this pH effect having been neglected in nearly all. Tryptophane was found to have a sharp narrow band at 2900 Ångström units and a broad region from 2850 to 2650 Ångström units, its width depending on the concentration of the solution. This checks the values of Stenström and Reinhard, a narrow band at 2875 and a broad one at 2750 Ångström units.

Of the aliphatic amino acids, cystine with an end of absorption as high as 2500 Ångström units is the only one which may interfere when present in normal amounts. The remainder transmit to the region near 2200 Ångström units under these conditions. Fortunately, cystine may be removed by reduction to cysteine whose absorption is much farther in the ultra-violet. Thus phenylalanine of all the amino acids has an absorption spectrum peculiar to itself, which may be used for its identification.

Of the aromatic amino acids phenylalanine exhibits relatively the weakest absorption. The ratio of absorbing strength for tryptophane, tyrosine, and phenylalanine is 1.0, 0.50, and 0.05, respectively. This ratio was obtained by determining the parts by weight of each required for distinctly characteristic absorption in a given amount of solution in a standard cell. Thus, unfortunately, tryptophane and tyrosine will interfere with the phenylalanine spectrum even if present in much smaller amounts. This interference was studied by taking standard solutions of phenylalanine with distinct absorption and adding successive portions of each of the other two. It was found that 0.07 part by weight of tryptophane or 0.12 part of tyrosine was sufficient to destroy the usefulness of absorption as a test for phenylalanine. The plates thus obtained show a very beautiful shifting of the one type of spectrum into the other, and could be used as a standard for

estimating the ratio of acids present in the range covered. (See Figs. 1 and 2, Plate 1.)

Spectroscopic identification should be restricted to phenylalanine, both because of standard colorimetric tests for tryptophane and tyrosine, and because of the common type of absorption of the latter two. The quantities of tyrosine and tryptophane present must be minimal to prevent interference. Tryptophane is removed by acid hydrolysis and excessive tyrosine may be partially crystallized from a concentrated hydrolysate solution leaving a suitable form of the original protein for investigation.

*Applications*—Several proteins were investigated as a means of testing the method. Gelatin has no tryptophane, very little (0.01 per cent) tyrosine, and 1.4 per cent phenylalanine (11). It was therefore an ideal material for investigation. A 0.2 per cent solution had four distinct bands at 2680, 2645, 2585, and 2525 Ångström units, identical with those of phenylalanine. These bands were even more distinctly present in hydrolysate solution of the same material. 50 gm. of dried alfalfa were extracted for its protein, and 3.5 gm. of gray powder 12.1 per cent nitrogen, were obtained. This material gave positive results for tyrosine with nitroso- $\beta$ -naphthol (12) but unsatisfactory Hopkins-Cole tryptophane tests. The solution obtained from acid hydrolysis was examined spectroscopically. Two faint but sharp bands at 2680 and 2645 Ångström units were observed. These are identical with two of the bands of phenylalanine, and the spectrum itself is almost identical with that obtained from a solution of 0.023 per cent phenylalanine and 0.003 per cent tyrosine. We accept this as definite proof of the presence of phenylalanine in the proteins from alfalfa.

In a recent communication, Lavin, Northrop, and Taylor (13) report that pepsin has an absorption band at room temperatures in the region 2600 to 2900 Ångström units which at  $-100^{\circ}$  is "resolved into a number of sharp, narrow bands." It is highly probable that these bands are due to phenylalanine (possibly tyrosine or tryptophane) and are correlated with activity only so far as the amino acids themselves are. Kistiakowsky and Arnold (14) have shown that the spectral sharpness of benzene derivatives is greatly enhanced at low temperatures. The spectrographic identification of the aromatic amino acids should be far more efficient and delicate at liquid air temperatures.

The author wishes to express his indebtedness to Dr. J. B. Conant and Professor G. B. Kistiakowsky for their invaluable advice and assistance throughout this research.

#### SUMMARY

The ultra-violet absorption spectra of tryptophane, tyrosine, and phenylalanine have been determined.

The peculiar absorption of phenylalanine may be used for its identification in proteins or their hydrolysates if the tryptophane and tyrosine present are minimal.

This method has been applied to gelatin and proteins from alfalfa giving positive results for the presence of phenylalanine in each.

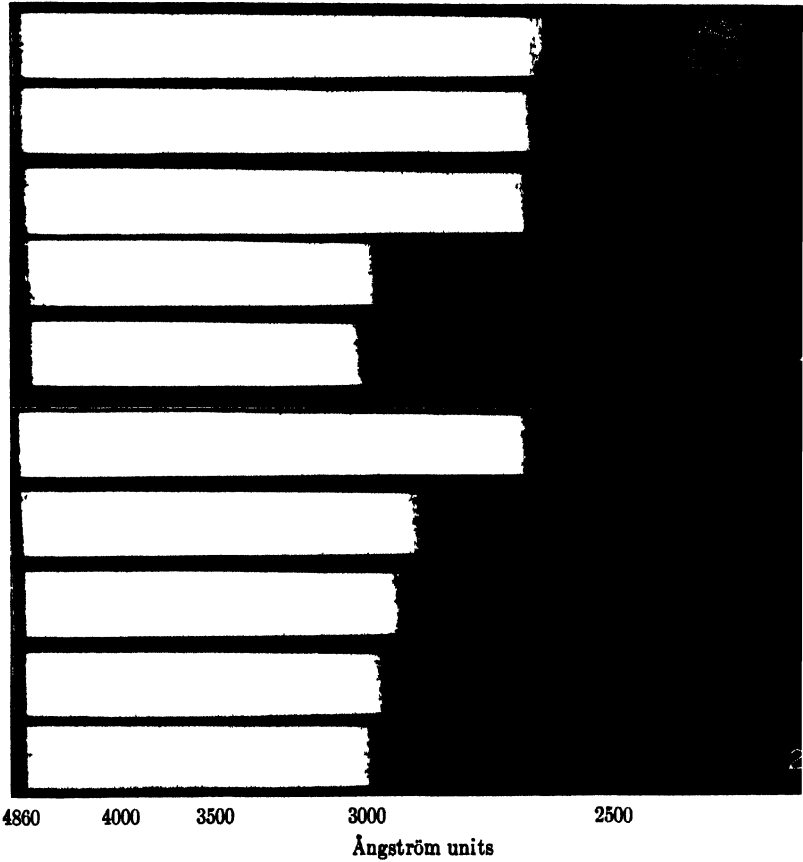
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#### EXPLANATION OF PLATE 1

FIG. 1. Spectra of phenylalanine and tyrosine. The first spectrum is for 0.023 per cent phenylalanine; to the remainder tyrosine has been added to the extent of 0.0008, 0.0015, 0.0031, and 0.0062 per cent, respectively.

FIG. 2. Spectra of phenylalanine and tryptophane. The first spectrum is for 0.023 per cent phenylalanine; to the remainder tryptophane has been added to the extent of 0.0008, 0.0015, 0.0031, and 0.0062 per cent, respectively.





# **STUDIES IN THE CARBOHYDRATE METABOLISM OF THE RABBIT**

## **I. THE TRUE BLOOD SUGAR VALUE IN CONVULSIONS DUE TO INSULIN ADMINISTRATION**

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(Received for publication, July 21, 1933)

Throughout the course of investigations on insulin there has arisen again and again the problem of why convulsions sometimes occur at blood sugar levels above 45 mg., and also, why they do not always occur at levels of 45 mg. or below. Penau and Simonnet (1) state that, "In the usual rabbit method, the appearance of convulsions does not necessarily coincide with the reduction of the blood sugar to 45 mgs. per cent." Harrop (2), discussing the toxic effects of insulin, says that he found large numbers of readings in diabetes of 75 to 45 mg. Most of these had no symptoms of an overdose of insulin. Clough, Allen, and Root (3) record 168 observations on rabbits in which the blood sugar was at or below 45 mg., although convulsions occurred in only 34.5 per cent of the animals. The highest value at which they reported convulsions to occur was 66 mg. and the lowest was 30 mg. They concluded, as have a number of workers, that the convulsive level is not a definite point *per se*. Forshay (4) has stated that the occurrence of reactions due to insulin shock bears no fundamental relation to the whole blood sugar concentration.

In all of this work on the blood sugar at convulsive levels, it has been the total reducing substances in the blood which were determined. It was the purpose of this research to determine the relative amounts of total and non-fermentable reducing substances in the blood at the incidence of convulsions due to insulin.

Rabbits were used as subjects. The animals were obtained from a dealer in the open market, and were not especially selected for uniformity. As the data for this paper were obtained from



another research which is in progress in the laboratory, the rabbits were subject to the following changes in diet. The rabbits were kept for 10 weeks on a mixed diet of oats, hay, greens, and water. This was followed by 10 weeks on a diet of alfalfa, molasses, hay, and water. This change in diet did not have any noticeable effect on the total or non-fermentable reducing substance in the blood. All the animals were fasted for 24 hours prior to use. The animals were bled, then injected with 2 units of insulin per kilo of body weight, and then bled again at the onset of convulsions. Lilly's insulin of serial No. F2-870007-V2 was used throughout. It was diluted with 0.9 per cent sodium chloride solution so that 1 cc. contained 2 units.

TABLE I  
*Summary of Results on 133 Observations*

Reducing substance	Mean value	Mean deviation	Mean deviation of mean
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
Initial, total.....	120	11.2	1.0
“ non-fermentable.....	36	6.6	0.6
“ fermentable (by difference).....	86	10.0	0.9
Convulsions, total.....	38	6.6	0.6
“ non-fermentable.....	36	5.8	0.5

Blood samples were obtained from the marginal vein of the ear. Hyperemia was induced by application of xylene. The blood was caught in a small dish which had been dusted with powdered potassium citrate.

The blood proteins were precipitated by the method of Folin and Wu (5) and the reducing substances in the filtrate were determined by the method described by Shaffer and Hartmann (6). The conversion table of Duggan and Scott (7) was used for determining the reducing substances of the sample in terms of glucose. The fermentable fraction was removed by the yeast method of Somogyi (8). The water obtained from centrifuging the yeast suspension was analyzed from time to time to be sure that no reducing substance was present. It was found that if the yeast cells were washed daily and kept in the refrigerator, there was no detectable reducing substance produced.

Somogyi's yeast method was checked by direct fermentation of blood by yeast at 30° for  $\frac{1}{2}$  hour. The results agree within 2 per cent.

The sensitivity of the blood sugar method at convulsive levels was checked by the addition of small amounts of glucose to blood at convulsive levels. In four tests in which 5 mg. per 100 cc. of

TABLE II

*Table of Fourteen Individual Observations*

Since the author is skeptical of results selected to substantiate a thesis, in this table every tenth observation is shown when the 133 observations were arranged in chronological order.

Initial sample		Sample during convulsions	
Total reducing substance	Non-fermentable reducing substance	Total reducing substance	Non-fermentable reducing substance
mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
123	41	29	26
129	44	41	31
131	38	34	34
121	34	34	34
124	31	37	34
115	32	34	32
126	38	41	37
130	43	48	48
135	41	41	40
105	33	36	33
136	45	39	36
131	36	33	36
113	27	28	26
134	33	34	33
Average . . . 125	35	36	34
$\epsilon$ . . . . . 9.1	5.5	5.3	5.5
$\epsilon_M$ . . . . . 2.4	1.5	1.4	1.5

blood were added, there was a recovery of 93 per cent of the added glucose. In three tests in which 10 mg. per 100 cc. of blood were added, there was a recovery of 96 per cent.

*Results*

The results of 133 determinations are summarized in Table I, and for the benefit of those who prefer individual results, 10 per cent of the observations are given in detail in Table II.

In measuring precision, the mean deviation  $\epsilon = \pm\sqrt{2x^2/(N-1)}$  rather than the standard deviation  $\sigma = \pm\sqrt{2x^2/N}$  was used, for the reasons outlined by Scott (9). The mean deviation of the mean  $\epsilon_M = \epsilon/\sqrt{N}$  is shown in the final column.

According to the above results, when a rabbit is in convulsions due to insulin, the true glucose value is zero, within the precision of the method used. In this group of 133 determinations, the total reducing substance in convulsions due to insulin varied from 53 to 26 mg. The corresponding range found by Clough, Allen, and Root (3) was from 66 to 30 mg. In our rabbits the range for the non-fermentable fraction was from 50 to 26 mg. From these results it can be seen that the range of the non-fermentable fraction covers the usual convulsive ranges of total reducing substances with remarkable precision. It will be noticed that non-fermentable reducing substances at the initial and the convulsive levels are essentially identical in concentration and as measured by the respective deviations are relatively constant. This is in accord with the results of Somogyi (10) who stated that, "The amount of reducing non-sugars in human blood is found to be very uniform . . . It is independent of the blood sugar level." According to the above values, the non-fermentable reducing substance is unaffected by amounts of insulin sufficient to produce convulsions.

The evidence which is submitted here indicates that when a rabbit is in convulsions, whether the total reducing substance be 53 or 26 mg., the true sugar value is approximately zero. If this is true, the reason underlying the apparent variations in the blood sugar level during convulsions becomes apparent and these variations should largely disappear when the fermentable reducing power alone is considered.

#### SUMMARY

1. The total reducing substance in the blood at the incidence of convulsion is the same as the non-fermentable reducing substance in normal blood and blood during convulsions.
2. The non-fermentable reducing substance in rabbit blood is found to be independent of the total reducing values and is not affected by insulin in doses large enough to produce convulsions.

I am indebted to Professor E. L. Scott under whose direction this work was performed.

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## THE CHEMISTRY OF EMBRYONIC GROWTH

### IV. THE REQUIREMENT OF THE PIG EMBRYO FOR COPPER

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(Received for publication, January 10, 1934)

In a previous report (Wilkinson and Gortner, 1932) it was observed that the ash of the pig embryo at different stages of development, although subjected to the same treatment, was of different color. The ash from the embryos 10, 15, and 30 mm. long ranged from a bluish tint to a distinct blue. After the 30 mm. stage the blue faded toward white. The ashes of the embryos 50 to 110 mm. long were white, while the ashes of those 160 to 240 mm. long ranged from a faint pink to a brick-red. So characteristic were the colors that different stages of development in some instances could, in a rough manner, be recognized by the color of the ash. Particularly was this true of the embryos 30 to 40 mm. long, whose ash was a distinct blue. At that time we stated that the red color in the later stages was undoubtedly due to the iron oxide formed from the iron-hemoglobin complex, but the cause of the bluish tint in the earlier stages remained to be determined. We felt that the persistent bluish tint might represent evidence of a greater percentage of copper present in the ash of those earlier embryos.

The significance of copper in mammalian life has only recently been recognized.

In 1928, 1929, Hart, Steenbock, Waddell, and Elvehjem showed that anemia produced in the rat by a continuous milk diet was not cured when the diet was supplemented by iron. On the other hand, an addition of a trace of copper brought about an immediate recovery. Since then many papers have appeared concerning the biochemical and physiological aspects of copper in animal nutrition, *e.g.* Cunningham (1931), Orten, Underhill, and Lewis (1932),

Josephs (1932), all of which confirm the original idea of Steenbock *et al.* that iron in nutritional anemia has no permanent curative effect unless copper is also present. In pigs made anemic by continuous milk diet, Elvehjem and Hart (1932) reported that when pure iron was added to the whole milk diet there was a small but temporary improvement of the hemoglobin—but a rapid and complete recovery was obtained only after copper was added.

It appears from these results that copper is necessary for the formation of hemoglobin. If this be true, apparently the logical place to study the copper content of the mammal is in the embryonic stages, when red blood cell formation is taking place rapidly. Since it is known that the chief hematopoietic function resides in the liver of the embryo, our attention was naturally drawn toward that organ.

Concerning the occurrence of copper in the mammalian embryo, no comparative work has been done. In a few instances, in which general analyses have been made for copper, the embryo has been included. But such work was mainly to determine the ubiquity of copper, rather than any relationship it had to the developing mammal.

Loesche (1931) showed that the unincubated egg contains around 1.1 mg. of copper per kilo of fresh weight. As would be expected, 1 or 2 days before hatching the chick contains this same amount of copper. But the phenomenal occurrence is the concentration of the element in the liver, which is about 20 times greater than in the remainder of the organism.

The present experimental study was undertaken to determine whether the blue color of the ash was actually due to the copper present; to determine the amount of copper in the whole embryo at different stages of development; to study the amount of copper in the liver of the embryo and compare this amount to the amount found in the whole embryo.

### *Methods*

In so far as was possible the collected material was removed from the amniotic fluid within an hour, dried by filter paper, weighed, and immediately placed in silica crucibles for ashing in a muffle furnace, the temperature being slowly raised to 600° and held at this level for 12 hours. Upon removal and cooling the ashes

were examined for copper according to the method of Elvehjem and Lindow (1929) as modified by Cunningham (1931). This method is dependent upon the greenish yellow color produced by the copper-pyridine-thiocyanate complex. Once the color is formed, it is extracted with a definite amount of chloroform and comparisons are made with a suitable standard of similarly treated copper sulfate.

A method was also developed for the study of the individual organs of the embryo. It is a matter of common experience of one who has worked with early embryonic material that because of the gelatinous consistency a dissection is almost impossible. If, however, the embryos are frozen at 15°, the individual organs can be easily removed with a miniature dissection set. Once removed, they are allowed to soften on a watch-glass, the excess fluid removed by filter paper, and the organs weighed. In this manner, definite and clean cut dissections can be obtained of the various embryonic organs.

The data are shown in Table I.

#### DISCUSSION

The data obtained disclose the following points: (1) The percentage of copper with respect to the ash of the embryo increases to the 30 mm. stage and then consistently decreases. (2) The mg. of copper per kilo of fresh weight increase up to the 30 mm. stage and then decrease. (3) There is a continued increase in the amount of copper present per embryo.

It has been observed previously (Wilkerson and Gortner, 1932) that similar peaks occur in the percentage of sulfur, glutathione, and total nitrogen at the 30 mm. stage. From the data presented here, it seems more apparent than before that these peaks are relative rather than actual. The high points demonstrated in the curves when calculations are made with respect to the ash are no doubt due to the subsequent influx of calcium, phosphates, and iron which constitute the bulk of the ash after the 40 mm. stage. When calculations are made on the fresh weight, the apparent peak at the 30 mm. stage is due to the disproportion of the percentage gain in weight to the percentage gain in element or compound studied. It will be observed from Table I, in comparing the per cent copper increase per embryo to the per cent weight increase per



TABLE I  
Showing Copper Content of Whole Pig Embryo and Embryonic Livers at Different Stages of Development

	10 56 16.77	20 57 59.09	25 16 28.58	30 25 76.64	40 27 149	60 10 14.96	80 3 113.37	110 2 171.57	160 2 476.12
Length of embryo, mm.....									
No. of embryos.....									
Total weight, gm.....									
Ash, gm.....	0.134	0.454	0.237	0.568	0.133	1.63	1.57	2.37	6.63
Cu, mg.....	0.166	0.650	0.347	1.455	2.63	2.52	1.218	0.920	2.32
“ in ash, per cent.....	0.123	0.143	0.146	0.256	0.197	0.154	0.0775	0.0388	0.0356
“ per kilo, mg.....	9.89	11.00	12.16	19.02	17.42	16.84	10.70	5.36	4.87
“ “ embryo, mg.....	0.0029	0.0124	0.0216	0.0582	0.090	0.252	0.406	0.460	1.16
Ratio, weight of livers to total body weight, per cent.....	16.23	14.53	13.72	13.63	12.83	11.18	6.38	4.26	4.25
Ash in livers, per cent.....	0.116	0.122	0.120	0.119	0.124	0.134	1.26	1.30	0.115
Cu “ “ .....	0.541	0.559	0.572	0.559	0.561	0.551	0.578	0.53	0.544
“ per kilo, gm.....	0.0690	0.0685	0.0680	0.0667	0.0630	0.0740	0.0733	0.0694	0.0628
Amount Cu per liver, mg.....	0.0024	0.0125	0.0182	0.0465	0.0465	0.175	0.261	0.300	0.740
Total Cu in liver, per cent.....	100	100	84.25	89.89	71.72	69.44	64.28	65.21	63.79
Increase in weight per embryo, per cent.		251.1	69.52	71.91	80.00	171.5	152.60	126.99	152.28
“ “ Cu per embryo, per cent.....		327.10	89.47	155.55	54.63	180	61.11	133.00	152.17

embryo, that up to the 30 mm. stage the former is much greater than the latter, while after the 30 mm. stage, the latter is equal to and in most instances greater than the former.

In the embryo 10 mm. long the liver constituted 16.23 per cent of the total bulk, while in the embryo 160 mm. long the liver accounted for only 4.25 per cent of the entire weight. The ash of the liver and the copper content, calculated either on the basis of the ash or the mg. of copper per kilo of wet weight, were all experimentally constant. In the early embryos (10 to 20 mm.) practically all the copper is present in the liver, and the percentage of the total amount of copper present in the liver decreases as the embryo becomes older. After 80 mm., however, the amount of copper present in the liver is apparently constant. These results are to be expected, since in the embryo the liver is the chief hematopoietic organ and transfers this property gradually to the bone marrow as that substance develops and becomes functional.

We early suspected that the blue color of the ash was due to copper. This was demonstrated as follows:

In the embryo 40 to 60 mm. long a portion of the bones has already begun to calcify. During this process endochondral ossification takes place in the hyaline cartilage; the latter gradually undergoes degenerative changes, disappears, and is replaced by bone. This process is not a simple metaplastic transformation of the cartilage to bone but is a development of an entirely new tissue which replaces the cartilage. This process begins around the 30 to 40 mm. stage. If embryos about 40 mm. in length were placed in a muffle furnace and the temperature allowed to rise gradually, it was observed that after a short period the embryo was completely submerged in its own fluid which had exuded from the body during heating. Upon continued heating it was found that before ashing was complete the skeletal structures were still present intact, but in a dried condition. If heating was continued, but not to complete ashing and crumbling, it was observed that the uncalcified portions of the skeleton acquired a distinct blue color. Even in individual bones which were only partly calcified, that portion which was still hyaline was colored blue. This blue material was easily separated by means of crystal forceps from the other skeletal structures, and upon analysis it was found that the bluish colored hyaline ash contained practically all the

copper, the remaining skeleton practically none. If, however, the livers were removed from the embryos before ashing, no blue coloration of the hyaline material was observed and its ash contained no copper. This indicated two things: that the hyaline embryonic cartilage has a specific affinity for copper in solution, and that the blue color is actually due to copper.

#### SUMMARY

1. There is a constant increase in copper throughout the embryonic period and, therefore, a demand for copper by the growing embryo.

2. Relatively, there is a continual increase in copper up to the 30 mm. stage following which there is a steady decrease. It is suggested that these trends are relative rather than absolute, and are due to a more rapid influx of calcium, phosphates, and iron (as well as organic matter) following the 30 mm. stage.

3. The ratio of total weight of the liver to the total weight of the embryo has been determined, and it has been found that the liver of the embryo 10 to 20 mm. long, representing 14 per cent of the total body weight, contains practically 100 per cent of the total copper of the body, while the liver of the embryo 160 mm. long, representing only 4 per cent of the total body weight, contains about two-thirds of the total copper in the body. This is thought to be associated with the hematopoietic function of the liver in the embryo.

4. The percentage of copper in the livers and the liver ash was found to be constant throughout.

5. The blue color of the ash in the early stages of embryonic growth is due to the relatively greater amount of copper present in the ash. There is also an affinity of the hyaline cartilage of the embryo for the copper in solution.

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## THE ERGOT ALKALOIDS

### II. THE DEGRADATION OF ERGOTININE WITH ALKALI. LYSERGIC ACID

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(Received for publication, January 22, 1934)

About 2 years ago Smith and Timmis<sup>1</sup> recorded the important observation of the formation of a basic degradation product, ergine, by the action of methyl alcoholic alkali on the ergot alkaloids, ergotinine and ergotoxine. From the analysis of ergine and a number of its salts, they derived for it the formula  $C_{17}H_{21}ON_2$ . In the course of a systematic investigation of the effect of various hydrolytic procedures on ergotinine while confirming the formation of ergine by the procedure employed by Smith and Timmis, we have observed the formation in better yield of a new degradation product when aqueous alkali is used.

In preliminary experiments it was found that crystalline ergotinine could be refluxed as a suspension in 6 per cent aqueous potassium hydroxide without appreciable alteration. This apparent resistance proved to be due to its insolubility. If ergotinine is first rapidly dissolved in methyl alcoholic potassium hydroxide and the solvent is immediately removed at low temperature and pressure, a resinous residue remains. When this residue is heated with aqueous alkali, as described later, it gradually dissolves with the liberation of ammonia due to cleavage of the amide group of ergotinine. Naturally, no ergotinine could be recovered from this reaction mixture. Furthermore, none of the base, ergine, could be detected on direct extraction of the alkaline hydrolysate. However, a new substance was obtained in good yield on gentle acidification, which possessed both acid and basic properties and which we have named *lysergic acid*. This acid is optically active ( $[\alpha]_D^{20} = +40^\circ$  in pyridine) and crystallizes in beautiful leaflets.

<sup>1</sup> Smith, S., and Timmis, G. M., *J. Chem. Soc.*, 763 (1932).

The analytical data obtained with the acid indicate a formula  $C_{16}H_{16}O_2N_2$ . This is supported by the analysis of its *methyl ester* which was obtained with diazomethane. The acid has no methoxyl group but still possesses the original N-methyl group of ergotinine. Titration showed the presence of one carboxyl group. It still gives the characteristic blue Keller reaction of the original alkaloid.

The comparative accessibility of this degradation product in workable yield from ergotinine makes it an important object for further study. An attempt to determine its structure is therefore in progress.

The natural thought occurred that the failure to recover ergine from the above reaction mixture might have been due to the fact that although it could have been formed during the reaction in aqueous alkali it might have been further degraded to lysergic acid. In order to determine this point we have replaced ergotinine in the above procedure by ergine. Although a crystalline acid was obtained in small yield it appeared on analysis to be definitely different from lysergic acid. With the amount available it was possible to give it but preliminary study. A report of this work will be left to a later occasion.

When the above crude mother liquor from lysergic acid was continuously extracted with ether it was found that other acid material was formed in the reaction. From the ether extract, which smelled faintly of isobutyric acid, a crystalline ammonium salt was obtained which on analysis gave figures which approximated those of the salt of isobutyryl formic acid. This was definitely confirmed by its decomposition into isobutyric acid and by the formation of the phenylhydrazone of isobutyryl formic acid.

Isobutyryl formamide was first obtained by Barger and Ewins<sup>2</sup> by the destructive distillation of ergotinine. This experiment we have also repeated. The formation of isobutyryl formic acid itself, however, by what appears to be a hydrolytic cleavage under the influence of alkali may be significant. It is still premature, however, to attempt to interpret its mode of linkage in the molecule. Although probable, it is not as yet certain that the amide group present in ergotinine itself is the same as that which emerges as isobutyryl formamide on destructive distillation.

<sup>2</sup> Barger, G., and Ewins, A. J., *J. Chem. Soc.*, **113**, 235 (1918).

## EXPERIMENTAL

1 gm. of ergotinine was dissolved in 20 cc. of N-methyl alcoholic potassium hydroxide and the methyl alcohol was removed at once by distillation at low pressure. The residue was treated with 20 cc. of an 8 per cent aqueous solution of potassium hydroxide and the mixture was heated on the steam bath for 1 hour. A stream of nitrogen was passed through the flask during the heating and basic volatile material from the reaction mixture was collected by passing the gas through a solution of dilute hydrochloric acid.

After the reaction was completed, the hydrochloric acid solution following evaporation gave a residue of 75 mg. which proved to be ammonium chloride. The theoretical weight for 1 mole of  $\text{NH}_4\text{Cl}$  is 88 mg.

The alkaline solution was made acid to Congo red with sulfuric acid. At this point a considerable amount of partly crystalline material precipitated. The acid suspension as such was placed in an extractor and exhaustively extracted with ether. This ether extract was worked up as given below for isobutyryl formic acid. The aqueous suspension which remained was then filtered. The dark colored solid was treated successively with two 20 cc. portions of ammoniacal ethyl alcohol which left a residue which was inorganic. The filtrate on evaporation to dryness under reduced pressure gave a residue which was digested a short time with 5 cc. of methyl alcohol to remove colored impurities. After cooling, the undissolved crystals were collected. 0.26 gm. of a slightly colored crystalline solid was obtained which melted with decomposition at  $235^\circ$ .

Lysergic acid (although rather sparingly soluble) can be recrystallized best from water. It crystallizes as very thin hexagonal leaflets which melt with decomposition at  $238^\circ$ . The melting point varies somewhat with the rate of heating. Repeated recrystallization failed to raise the melting point. It separates with approximately 1 mole of water of crystallization. This water is held very tenaciously and can be removed completely only on drying at  $140^\circ$  and 2 mm.

$[\alpha]_D^{25} = +40^\circ$  ( $c = 0.500$  in pyridine)

$\text{C}_{16}\text{H}_{18}\text{O}_7\text{N}_2 \cdot \text{H}_2\text{O}$	Calculated, $\text{H}_2\text{O}$ 6.29; found, 6.12
$\text{C}_{16}\text{H}_{18}\text{O}_7\text{N}_2$	Calculated. C 71.69, H 6.00, N 10.45, $\text{CH}_2$ 5.60
Found.	" 71.84, " 6.03, " 10.87, " 4.92
"	" 71.69, " 5.93, " 10.50

Lysergic acid gives the characteristic blue Keller test of the ergot alkaloids. In dilute sulfuric acid solution it gives with benzaldehyde and sulfuric acid a violet-blue ring which rises through the solution. With diazobenzenesulfonic acid a brown amber color is given in carbonate solution. With alkaline nitroprusside a yellow is first formed which gradually changes to a yellow-green.

Lysergic acid behaves both as an acid and a base. It is soluble in sodium hydroxide, ammonium hydroxide, sodium carbonate, and hydrochloric acid. It is but slightly soluble in dilute sulfuric acid. It is sparingly soluble in the usual neutral organic solvents but is quite soluble in pyridine.

On titration with alkali against phenolphthalein, the acid does not give a sharp end-point. However, the results indicated a neutralization equivalent of approximately 300 when the air-dried substance was used. Material dried at 140° was not suitable for titration because of appreciable discoloration which interfered with the detection of the end-point. 12.735 mg. of substance were dissolved in 10 cc. of hot water and the solution was titrated against phenolphthalein with 0.1 N sodium hydroxide. Calculated for 1 equivalent, 0.489 cc.; found, 0.415 cc.

*Lysergic Methyl Ester*—Lysergic acid suspended in dry acetone slowly dissolved after the addition of diazomethane. The ester crystallized from benzene in thin leaflets which melted at 168°. It is soluble in ether, acetone, alcohol, and hydrochloric acid. It is insoluble in ammonium hydroxide and petroleum ether.

$C_{17}H_{15}O_2N_2$ .	Calculated.	C 72.34,	H 6.41,	N 9.92,	OCH <sub>3</sub> 11.00
	Found.	" 72.40,	" 6.19,	" 9.66,	" 11.95
		" 72.16,	" 6.35,	"	11.98

The ethereal extract of the original acidified alkaline reaction mixture was dried with sodium sulfate and concentrated under reduced pressure. About 0.2 gm. of an oil remained which smelled distinctly of isobutyric acid. It was completely soluble in water but not in petroleic ether.

A solution of the oil in 1 cc. of absolute alcohol was saturated with dry ammonia. On careful addition of ether crystals separated. After chilling the colorless material was collected. 50 mg. were obtained. It melted at 175° with considerable sublima-

tion before melting. A solution of the salt in 80 per cent sulfuric acid, although odorless at first, developed a distinct odor of isobutyric acid on warming. The salt was analyzed as such without attempting recrystallization.

$C_8H_{11}O_3N$ .	Calculated.	C 45.1,	H 8.28
	Found.	" 46.50,	" 8.99

These analytical figures thus only approximated those required by the ammonium salt of isobutyryl formic acid and because of the limited amount of the material available the phenylhydrazone was prepared for characterization as follows: The ammonium salt was dissolved in 0.5 cc. of water and an equivalent of phenylhydrazine dissolved in 0.5 cc. of acetic acid was added. After a few minutes heating on the steam bath the solution was carefully diluted. The phenylhydrazone crystallized on chilling. On recrystallization from dilute alcohol it formed long needles which melted at  $152^\circ$ . This is higher than the figures given by Tschtschenke ( $128^\circ$ )<sup>3</sup> and Tschelinzeff and Schmidt ( $143^\circ$ ).<sup>4</sup> Our substance was readily soluble in dilute ammonia and was reprecipitated in crystalline form on reacidification.

$C_{11}H_{14}O_3N_2$ .	Calculated.	C 64.08,	H 6.84,	N 13.59
	Found.	" 64.50,	" 6.86,	" 13.47
	"	" 64.32,	" 6.67,	" 13.72

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<sup>3</sup> Tschtschenke, D., *Bull. Soc. chim.*, series 4, **37**, 623 (1925).

<sup>4</sup> Tschelinzeff, W., and Schmidt, W., *Ber. chem. Ges.*, **62**, 2210 (1929).





# CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS

## III. THE CHEMISTRY OF OSTREASTEROL

By WERNER BERGMANN

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(Received for publication, January 24, 1934)

In a previous communication (1) the author has described the isolation and the properties of a new sterol, ostreasterol, which occurs in the oyster and other mollusks. Ostreasterol contains two double bonds and in analogy to the formula of other zoosterols the formula  $C_{27}H_{44}O$  was attributed to it.

When subjected to catalytic hydrogenation, ostreasteryl acetate adds 2 molecules of hydrogen. The hydrogenated product does not give a Liebermann-Burchard reaction and must therefore be considered to be saturated. The determination of the equivalent weight of ostreastanol acetate by saponification, a method which, as Sandquist (2) has shown, gives very accurate results, gave values which agreed very well with the formula  $C_{29}H_{47}O \cdot CO \cdot CH_3$ . Consequently the formula for ostreasterol has to be changed from  $C_{27}H_{44}O$  to  $C_{29}H_{48}O$ . The properties of ostreastanol acetate as well as those of ostreastanol, obtained by saponification of the acetate, were found to be very similar to those reported for sitostanol and sitostanol acetate.

The melting points and optical rotations of sitostanol and its derivatives, however, have been recorded differently in the publications of different authors. Ostreastanol was therefore compared side by side with a pure sitostanol.<sup>1</sup>

In addition to that, the acetates, phenylurethanes, and ketones were also compared (Table I). The properties of ostreastanol and its derivatives were identical with those of sitostanol and its derivatives. Mixtures of the corresponding substances did not show a

<sup>1</sup> The author is highly indebted to Professor R. J. Anderson for the gift of a sample of sitostanol.

depression of the melting point. The ostreastanol must be considered therefore as identical with sitostanol.

In the first paper on ostreasterol a tribromoostreasteryl acetate was described and it was suggested that the compound might consist of a mixture of 1 molecule of a dibromide and 1 molecule of tetrabromide. This seems indeed to be the case, for if bromine is added in a larger excess and the mixture kept standing for a longer time, a well crystallized bromide can be obtained, which melts at 150.5° and on analysis yields a tetrabromide  $C_{31}H_{50}O_2Br_4$ . The isolation of this product furnishes definite proof that ostreasterol

TABLE I  
*Comparison of Ostreastanol, Sitostanol, and Some of Their Derivatives*

	Ostreastanol		Sitostanol			
			Data found by author		Data recorded in literature	
	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$	M.p.	$[\alpha]_D$
	°C.	degrees	°C.	degrees	°C.	degrees
Sterol.....	141	+23.73	140-141	+23.53	137 (3), 144 (4), 142-143 (5)	+27.9 (3), +28.0 (4), +23.55 (5), +23.61 (5)
Acetate.	137	+14.58	137	+14.28	132 (3), 138 (5), 141 (6)	+12.72 (6), +14.41 (5)
Phenylurethane ....	175		174-175			
Ketone.....	157	+41.95	157	+41.75	157 (3)	+45.7 (3)

The figures in parentheses refer to the bibliography.

contains two double bonds. Ostreasterol is isomeric with stigmasterol. On catalytic reduction, however, stigmasterol yields stigmastanol which melts at 134-134.5°; hence a difference in the structure of the two sterols is clearly indicated.

Cholesterol has always been regarded as the typical sterol of the animal kingdom. The fact that ostreastanol is identical with sitostanol must be regarded as very significant because this is the first time that a sterol obtained from animal sources has been converted into a typical sterol of plant origin. It raises the question, therefore, whether a strict line can be drawn at all between zoosterols and phytosterols.

It seems possible that certain mollusks are unable to synthesize cholesterol, but that they use directly or in dehydrogenated form the phytosterols of their food which consists mainly of algæ and diatoms. It is further of interest to note, that in regard to Schoenheimer's work (7), which shows that sitosterol is not resorbed by higher animals, it seems almost certain that ostreasterol will not be resorbed either. As far as the sterols are concerned, the oyster and other mollusks would resemble a vegetable rather than an animal food.

#### EXPERIMENTAL

*Catalytic Hydrogenation of Ostreasterol Acetate*—3.25 gm. of ostreasterol dissolved in 100 cc. of glacial acetic acid were hydrogenated at 70° with 1 gm. of platinum black as catalyst. After 4 hours shaking, 200 mg. of platinum oxide were added. The mixture was shaken for 2 more hours, after which time 100 mg. of platinum oxide were added and the shaking continued for another hour. After that time 15.15 mg. of hydrogen had been absorbed corresponding to 2.23 double bonds. The hydrogenated product did not give a positive test for the Liebermann-Burchard reaction.

The mixture was now filtered hot from the platinum black and concentrated *in vacuo* to about 50 cc. After cooling the ostreastanol acetate crystallized out. It was recrystallized four times from alcohol. Ostreastanol acetate crystallizes in oblong plates and parallelograms. Its melting point is 137°.

*Rotation*—0.1025 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of +0.498°; hence  $[\alpha]_D^{19} = +14.58^\circ$ .

Mixed with sitostanol acetate, m.p. 137° and  $[\alpha]_D^{19} = +14.28^\circ$ , it did not show a depression of the melting point.

*Determination of Equivalent Weight*—Ostreastanol acetate was refluxed with approximately 0.1 N alcoholic potassium hydroxide for 1 or 2 hours and the excess of KOH titrated with 0.1 N HCl (see Sandquist (2)).

The average equivalent weight of three determinations was 457.2. Calculated for  $C_{29}H_{51}O \cdot CO \cdot CH_3$ , 458.4.

*Analysis*—4.541 mg. gave 13.505 mg.  $CO_2$  and 4.81 mg.  $H_2O$   
Calculated for  $C_{29}H_{51}O_2$ . C 81.15, H 11.87  
Found. " 81.13, " 11.85

*Ostreastanol (Sitostanol)*—The acetate was saponified in the usual manner and the sterol recrystallized three times from alcohol, m.p. 141°.

*Rotation*—0.1039 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of +0.82°; hence  $[\alpha]_D^{19} = +23.72^\circ$ .

Mixed with sitostanol, m.p. 140–141° and  $[\alpha]_D^{19} = +23.53^\circ$ , it melted at 140–141°.

*Ostreastanol (Sitostanol) Phenylurethane*—To a solution of ostreastanol in dry benzene, phenyl isocyanate was added in excess and the mixture was refluxed for 2 hours. The solvent and the excess of phenyl isocyanate were removed *in vacuo* and the residue dried *in vacuo* at 100°. It was then dissolved in chloroform, the solution filtered, mixed with alcohol, and concentrated until crystallization began. The urethane was recrystallized four times from alcohol, till the melting point was constant at 175°.

*Analysis*—2.95 mg. gave 0.066 cc.  $N_2$  at 25° and 761 mm.

Calculated for  $C_{28}H_{48}O_2N$ , N 2.61; found, N 2.56

The phenylurethane of sitostanol was prepared in the same manner. It melted at 174°. Mixed with ostreastanol phenylurethane it melted at 174–175°.

*Ostreastanone (Sitostanone)*—Ostreastanol was oxidized to ostreastanone by following the directions given by Windaus and Rahlen (3) for the preparation of sitostanone, m.p. 157°.

*Rotation*—0.0634 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of +0.885°; hence  $[\alpha]_D^{19} = +41.95^\circ$ .

Mixed with sitostanone, m.p. 157° and  $[\alpha]_D^{19} = +41.75^\circ$ , it did not show a depression of the melting point.

*Tetrabromoostreasteryl Acetate*—To a solution of 1 gm. of ostreasteryl acetate in 10 cc. of dry ether 20 cc. of a 5 per cent solution of bromine in glacial acetic acid were added. After 12 hours standing in the ice box a colorless precipitate had separated, which was filtered and recrystallized twice from a mixture of ethyl acetate and alcohol and twice from acetone. The tetrabromide crystallized in fine needles, m.p. 150.5°. When reduced with zinc dust in glacial acetic acid, it gave back ostreasteryl acetate.

*Analysis*—3.594 mg. substance gave 1.507 mg. Br

Calculated for  $C_{28}H_{46}O_2Br_4$ , Br 41.30; found, Br 41.93

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## **THE BLOOD CHEMISTRY OF SWINE**

### **I. BLOOD CHANGES FOLLOWING THE INGESTION OF GLUCOSE**

**BY DONALD F. EVELETH**

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*(Received for publication, October 13, 1933)*

The changes in concentration of constituents other than glucose, that occur during the absorption and utilization of this sugar, have not been studied in very great detail. It has been shown by several authors that there is an intimate relationship between the concentration of glucose and inorganic phosphate in the blood. McCullagh and McCullagh (1) have recently summarized this literature and shown experimentally that after the peak of glucose concentration in the blood, there is a marked diminution in the phosphate concentration, while relatively minor changes occur in the serum calcium. Mosenthal and Bruger (2) found that, in normal individuals after the ingestion of glucose, there was a fall in both urea and total non-protein nitrogen. Powers and Reis (3) showed that insulin reduced the amino acid concentration of the blood with little or no effect on the urea. During the course of some carbohydrate tolerance studies on pigs, the urea nitrogen, amino acid nitrogen, inorganic phosphate, and plasma calcium have been determined.

#### **EXPERIMENTAL**

Normal pigs, weighing from 40 to 60 pounds, were fasted approximately 16 hours before each experiment and were allowed at least 1 week's rest between experiments. Blood samples were obtained by amputation of about 1 inch of the tail. Hemorrhage was controlled by means of a rubber band wrapped tightly around the tail. Subsequent samples were drawn by removing the band and allowing the blood to flow from the coccygeal arteries. The pigs were placed on a table with holes for the legs, and held there by means of straps. It was found that pigs handled in this manner



did a minimum of struggling. A dose of 0.8 gm. of glucose per pound, dissolved in water to make a 60 per cent solution, was given by means of a stomach tube.

The blood was collected in test-tubes containing 50 mg. of sodium citrate per 10 ml. of blood. Glucose was determined by

TABLE I  
*Blood Changes Following Ingestion of Glucose*

Constituent	Time	Control group			Experimental group		
		Maximum	Minimum	Average	Maximum	Minimum	Average
	min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Glucose	0	52.6	40.0	49.2	74.0	42.8	55.9
	30	56.6	35.0	46.6	166.0	72.7	106.8
	60	55.0	40.2	48.3	307.7	95.0	142.3
	120	55.5	43.0	49.6	121.2	65.0	89.0
	240	60.0	49.0	54.6	86.9	31.2	65.8
Inorganic P	0	5.52	5.12	5.39	6.12	4.31	5.33
	30	5.00	4.63	4.88	5.59	3.33	4.63
	60	5.12	4.50	4.64	4.65	3.05	4.02
	120	5.33	4.59	5.01	7.02	3.68	4.78
	240	5.71	5.33	5.62	6.06	3.75	4.91
Amino acid N	0	9.52	7.77	8.92	9.46	8.00	8.76
	30	9.26	7.82	8.69	9.65	8.75	9.10
	60	9.31	7.62	8.75	10.77	8.97	9.76
	120	9.39	7.58	8.89	10.37	7.77	9.39
	240	9.75	8.00	9.16	9.33	7.36	8.43
Urea N	0	8.40	8.00	8.15	9.34	8.00	8.97
	30	7.81	7.63	7.75	9.35	7.63	8.89
	60	8.43	7.69	7.90	9.82	7.54	8.88
	120	8.84	7.63	8.30	9.82	7.32	8.94
	240	8.40	8.00	8.33	9.80	7.79	8.84
Serum Ca	0	11.17	8.40	10.60	11.13	8.65	10.08
	30	10.02	8.75	9.90	10.90	8.91	10.17
	60	10.35	8.75	9.96	10.47	8.84	9.76
	120	10.76	8.61	9.75	10.99	8.68	9.93
	240	10.52	8.37	10.21	10.78	8.11	9.41

Benedict's (4) method, a Folin-Wu (5) filtrate being used, amino acid by Folin's (6) technique, calcium by the gasometric method of Van Slyke and Sendroy (7), inorganic phosphate by the Fiske and Subbarow (8) method, and urea by direct Nesslerization of a sodium tungstate sulfosalicylic acid filtrate (9).

## DISCUSSION

The maximum, minimum, and average values obtained in nine experimental and four control periods are given in Table I.

TABLE II  
*Effect of Ingested Glucose on Blood of Pig 19*

Constituent	Time	Control	Experimental
	<i>min.</i>	<i>mg. per cent</i>	<i>mg. per cent</i>
Sugar	0	52.6	46.0
	30	50.8	133.3
	60	52.6	95.0
	120	55.5	76.9
	240	49.0	72.5
	300		62.5
Inorganic P	0	5.12	5.71
	30	4.92	5.59
	60	5.26	4.65
	120	5.33	3.81
	240	5.33	5.00
	300		4.00
Amino acid N	0	9.00	8.78
	30	8.26	8.79
	60	8.34	10.00
	120	8.26	9.39
	240	8.46	8.00
	300		9.10
Urea N	0	8.00	8.54
	30	7.63	8.75
	60	7.82	9.05
	120	7.63	7.63
	240	8.00	7.79
	300		8.70
Calcium	0	10.00	8.65
	30	9.90	9.65
	60	9.64	8.84
	120	9.20	8.78
	240	9.21	8.95
	300		9.35

The same animals were used for experimental and control subjects, with eight individual pigs being used. In Table II are given the data obtained in the control and glucose feeding experiments on Pig 19.

The metabolism of sugar by pigs seems to be subject to considerable individual variation. Hewitt (10), using the Folin-Wu method, has found a variation of from 38 to 500 mg. per cent with an average of 128 mg. per cent for the blood of normal swine. In analyses made on various fasting pigs, during the course of some other investigations, blood sugar values of from 30 to 280 have been encountered. In general, it seems that the older pigs tend to have higher blood sugar levels. Biester (11) has reported a case of diabetes in a pig as a result of infection in the pancreas. Carlson and Drennan (12) showed that a normal pig had very low carbohydrate tolerance. In neither of these investigations were blood sugar values determined but urine analyses were carried out.

In the experiments reported here, the blood sugar in the fasting pigs varied between 40 and 74 mg. per cent. In only one case did the blood sugar go higher than 300 after the ingestion of glucose. This pig has no history of sickness and subsequent glucose tolerance tests have shown an average rate of removal of sugar from the blood. Individual pigs show some variation in the rate of metabolism of glucose. In some cases, the maximum concentration occurred in 30 minutes while in others the blood sugar continued to increase for an hour or more. The peak of the amino acid curve usually occurred about 30 minutes after the peak of the sugar curve, but in certain cases the two peaks were simultaneous. The urea usually increased slightly but the average values show practically no change. This is probably due to the difference in rate of response. The inorganic phosphorus decreased in all experiments with a definite low point about 30 minutes after the peak of the sugar curve. Here there are definite individual differences. Urea and calcium, in general, tended to rise following the ingestion of glucose with a subsequent decrease.

In comparing these values with others reported in the literature, it must be borne in mind that all of these analyses were made on arterial blood.

#### SUMMARY

Data are given which indicate that the ingestion of glucose produces a series of changes in the concentration of urea, amino acid, inorganic phosphate, and calcium of the blood of the pig.

The author is indebted to Mr. Howard C. Nowlin for valuable assistance, particularly in the bleeding of the pigs.

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# STUDIES OF THE ACID-BASE BALANCE OF THE BLOOD\*

## I. A MICROTECHNIQUE FOR THE DETERMINATION OF THE ACID-BASE BALANCE OF THE BLOOD

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For the study of the acid-base balance of the blood in small animals and in the arterial blood of humans requiring frequent observations, for which arterial punctures are inadvisable, the following micromethod has been devised.

In this method, with a sample of 0.1 cc. of whole blood, the percentage of red cells is determined by a hematocrit reading, the pH colorimetrically by the method of Hastings and Sendroy (2), and the total CO<sub>2</sub> content by the method of Van Slyke and Neill (4). From these data, the bicarbonate content of the serum is calculated, making it possible completely to characterize the acid-base condition of the blood.

### *Method*

*Apparatus*—An acid-base pipette was developed for the micro-method. The pipette consists of a lower stem (2, A, Fig. 1), graduated from 0.0 to 0.1 cc. in 0.01 cc. divisions, and a bulb (1, A, Fig. 1). The bulb has an inside diameter of approximately 11 mm. The essential characteristic is that this bulb be of the same diameter and of the same kind of glass as the tubes containing the Hastings-Sendroy bicolor pH standards. In this laboratory the acid-base pipettes are made by sealing 0.2 cc. serological pipettes to test-tubes similar in glass and diameter to those used for pH standards, and then drawing down the upper end. The serologi-

\* This work was supported by a financial grant from the Julius Rosenwald Fund.

cal pipette is cut off at the 0.1 cc. mark and the end beveled by grinding, as shown in Fig. 1. The entire pipette, which is about 17 mm. long, is accurately calibrated to contain 2.0 cc. by weighing mercury.<sup>1</sup> Large rubber bands are needed for closing the pipettes; Eberhard Faber No. 84 proved quite satisfactory.

Receivers for collecting the blood samples are also made by sealing off the bottom of 1½ inch funnels, as shown in Fig. 1.

A set of pH standards must be prepared according to the directions of Hastings and Sendroy (2).

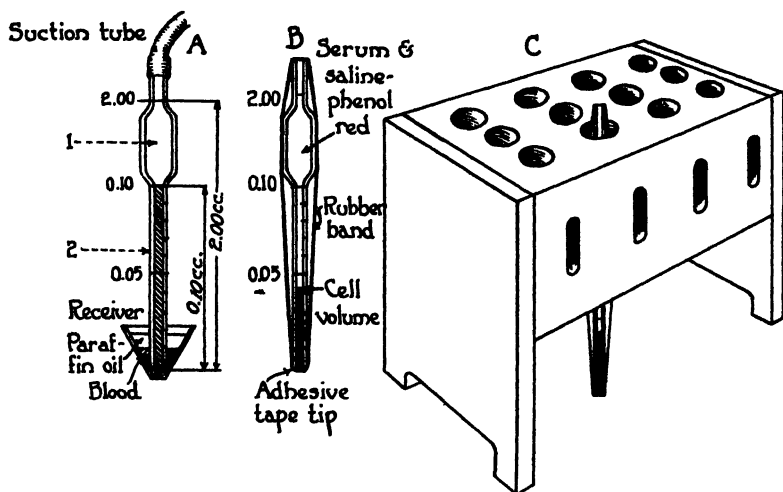


FIG. 1. A shows the method of filling the micropipette for the determination of acid-base balance; B, pipette after centrifuging, ready for reading  $V_c$  and pH.; C, comparator block for reading pH, with Hastings-Sendroy bicolor standards, with a pipette in place.

A comparator block (C, Fig. 1) in which the holes across one row are drilled clear through, so that the graduated stem of the pipette extends through, permitting the color in the bulb to be compared with the pH standards, must also be prepared. For convenience, legs may be added as shown.

<sup>1</sup> Acid-base pipettes of such design may be obtained from the Fisher Scientific Company or Arthur H. Thomas Company.

*Solutions Required*

1. Saline-phenol red solution. 9.00 gm. of NaCl are dissolved in freshly distilled water in a liter flask. 105.25 cc. of 0.0075 per cent phenol red solution made from 0.1 per cent phenol red solution according to Hastings and Sendroy (2) are added and the mixture is diluted to the 1 liter mark.
2. Normal saline. A 0.154 M solution of NaCl in distilled water without phenol red is prepared for control tubes.
3. Neutral paraffin oil.
4. Paraffin oil saturated with a gas mixture of 5 per cent CO<sub>2</sub> and 95 per cent O<sub>2</sub>.
5. Potassium oxalate. A solution containing 5 gm. of potassium oxalate per liter is prepared. Extreme care must be used to get pure oxalate containing no carbonate or free acid.
6. Saturated NaOH solution.

*Technique*

Receivers for the blood are prepared by adding 0.2 to 0.3 cc. of the 0.5 per cent potassium oxalate solution to the sealed funnels and evaporating off the water in the oven at 110°.

Sufficient saline-indicator solution for the number of samples to be run on a given day (10 to 250 cc.) is first adjusted to pH 7.4. This is done by adding sufficient dilute NaOH to give the proper color. The dilute NaOH is prepared fresh each time by adding 1 drop of the saturated NaOH solution to 20 to 25 cc. of distilled water in a test-tube. After the pH of the indicator solution has been adjusted, the solution is covered with about an inch of neutral paraffin oil.

*Collection of Sample*—Blood is collected under paraffin oil previously saturated with 5 per cent CO<sub>2</sub> and 95 per cent O<sub>2</sub> to prevent loss of CO<sub>2</sub> and consequent change of pH. The finger, after being pricked with a needle or blood lance, is stuck into the oil contained in one of the receptacles, and the blood permitted to run under the oil. If necessary the finger may be gently pressed to facilitate the flow of blood. About 6 to 12 drops of blood are collected and thoroughly stirred to prevent clotting.

*Filling the Pipettes*—The pipettes are filled to the 0.1 cc. mark with blood, and then saline-phenol red indicator solution is drawn into the pipettes to the 2.00 cc. mark. The filled pipette, after



being tipped with a small strip of adhesive tape, is capped with a heavy rubber band and centrifuged for 1 hour at about 2500 R.P.M.

In filling the pipettes from the funnels, care must be exercised to prevent oil globules from being drawn into the pipette with the blood. This difficulty is minimized if the oil is prevented from flowing up into the capillary of the pipette as it is placed in the oil to reach the blood by maintaining a slight positive air pressure on the suction tube. When filling, the blood is drawn slightly past the 0.1 cc. mark before being removed from the funnel, and final adjustment of the amount of blood is carried out by drawing off the excess on a clean towel till exactly 0.1 cc. remains in the capillary. Three or four pipettes are filled for each sample. In addition one control tube is prepared by diluting 0.1 cc. of blood with the normal saline instead of the saline-indicator.

*Reading Percentage of Cells*—After centrifuging, the percentage of red blood cells ( $V_c$ ) is noted by reading the volume on the stem of the pipette, as in a hematocrit.

*Reading the pH*—The pH of the serum ( $pH_s$ ) is determined according to the method of Hastings and Sendroy by comparison with standard color tubes in a comparator block, with the control pipette prepared with normal saline instead of the saline-indicator solution to balance out the yellow color due to the serum. It is best to prepare a control pipette for each separate sample if this is at all possible, since the depth of color in different sera frequently varies considerably. The temperature at which the pH is read is recorded in order that the proper correction to give pH at 38° may be applied.

*Determination of CO<sub>2</sub> Content*—The entire 2.0 cc. sample of solution in the pipette is then transferred quantitatively to the extraction chamber of the Van Slyke manometric blood gas apparatus and its total CO<sub>2</sub> content determined by the method of Van Slyke and Neill (4). The transfer is made in the usual way under mercury, but no rubber tip is necessary for the pipette when ground as shown. After the solution from the pipette is in the extraction chamber, the pipette is rinsed out twice by drawing the lactic acid reagent up into it and allowing it to drain. The lactic acid reagent used consists of 2.25 cc. of distilled water plus 0.25 cc. of 10 per cent lactic acid plus 1 drop of caprylic alcohol, for each analysis.

After extraction of the gases in a 50 cc. volume, the pressure of

the extracted gases is read at the 0.50 cc. volume. The  $\text{CO}_2$  is then absorbed with 4 drops of 5 N NaOH and the pressure again read at 0.50 cc. The difference in the two pressure readings is multiplied by the appropriate factor, depending on the temperature (found in Table I), to obtain the total  $\text{CO}_2$  content of the blood plus the indicator solution used.

*Analysis of Indicator Solution*—After adjustment to pH 7.4, the total  $\text{CO}_2$  content of the saline-indicator solution is determined

TABLE I

*Table of Carbon Dioxide Factors*

$P \times f = (\text{CO}_2)$  in mm per liter, with 2.0 cc. samples in a 50.0 cc. Van Slyke manometric blood gas apparatus; the pressures are read at 0.5 cc. in mm. of mercury.

$a = 0.5 \text{ cc.}$ ,  $S = 2.5 \text{ cc.} + 2.0 \text{ cc.} = 4.5 \text{ cc.}$ ,  $A = 50.0 \text{ cc.}$ ,  $i = 1.037$ .

Temperature	Factor ( <i>f</i> )
°C.	
15	0.01601
16	0.01590
17	0.01580
18	0.01571
19	0.01562
20	0.01553
21	0.01545
22	0.01536
23	0.01527
24	0.01518
25	0.01510
26	0.01502
27	0.01494
28	0.01486
29	0.01478
30	0.01470

on a 2.0 cc. sample, with a regular Van Slyke pipette with stop-cock. The same volume of reagents is used in this analysis as was used in the blood determinations of  $\text{CO}_2$  above. Extraction is carried out at 50.0 cc. and pressures are read at 0.50 cc. as with the blood determinations. The difference in pressure before and after absorption of the  $\text{CO}_2$  by NaOH is multiplied by the appropriate factor from Table I as before, thus giving the total  $\text{CO}_2$  content of the saline-indicator in mm per liter.

**Determination of  $\text{CO}_2$  Content of Blood**—The  $\text{CO}_2$  content of the saline-indicator multiplied by 0.95 is subtracted from the  $\text{CO}_2$  content of the blood plus the indicator solution, and this result is multiplied by 20 to give the total  $\text{CO}_2$  content,  $(\text{CO}_2)_t$ , of the original blood sample in mm per liter of blood.

**Correction of pH, Values to  $38^\circ$** —From Table II the correction to be subtracted from pH, at  $t^\circ$  to give pH, at  $38^\circ$  may be obtained. This is the correction to be applied when the Hastings-Sendroy bicolor standards are prepared to be read at  $38^\circ$ .

Table II is obtained by assuming that the change in pH with change in temperature is linear between  $20$ – $38^\circ$  and by assuming

TABLE II

*Table for Correction of pH Read at  $t^\circ$  with Hastings-Sendroy Bicolor Standards Carrying  $38^\circ$  Label, to pH at  $38^\circ$*

$t$ °C.	Correction
18	−0.100
19	−0.095
20	−0.090
21	−0.085
22	−0.080
23	−0.075
24	−0.070
25	−0.065
26	−0.060
27	−0.055
28	−0.050
29	−0.045
30	−0.040

that the magnitude of the change in pH over this temperature range is 0.01 pH per degree of change in temperature, as reported by Cullen (1).

From the above determinations the following data are available: (1) percentage of red blood cells; (2) pH of the serum at  $38^\circ$ ; and (3) the total  $\text{CO}_2$  content of the whole blood in mm per liter of blood.

### Calculations

Table I shows the factors by which the pressure in mm. of mercury of the  $\text{CO}_2$  at a volume of 0.5 cc. is multiplied to give the

total  $\text{CO}_2$  content of the solution analyzed in mm of  $\text{CO}_2$  per liter, when 2.0 cc. samples are used for analysis. The values in Table I were calculated from the equations of Van Slyke and Neill (4), with the revised constants (5).

$\text{CO}_2$  (mm per liter of solution analyzed) =  $P \times f$  where  $P$  denotes pressure of  $\text{CO}_2$  in mm. of mercury and  $f$  is defined by the equation,

$$f = 0.01532 \times (1/(1 + 0.00384t)) \times (1 + 0.989\alpha')$$

Values for  $(1/(1 + 0.00384t))$  and for  $\alpha'$ , the Ostwald distribution coefficient for  $\text{CO}_2$ , are taken from Van Slyke and Neill (4).

Factors given in Table I are for use under the following conditions:  $\text{CO}_2$  pressures read at a volume of 0.5 cc. in a 50.0 cc. machine, 2.5 cc. of reagent in the analysis (2.25 cc. of distilled  $\text{H}_2\text{O}$  + 0.25 cc. of 10 per cent lactic acid + 1 drop of caprylic alcohol).

In addition to the determination of  $\text{CO}_2$  on the blood-saline-indicator mixture, a blank  $\text{CO}_2$  determination is made on 2 cc. of the saline-indicator alone. The  $\text{CO}_2$  content of the whole blood in mm per liter is calculated from the equation,  $(\text{CO}_2)_b = 20 ((\text{CO}_2)_{bl} - 0.95 (\text{CO}_2)_l)$ , where  $(\text{CO}_2)_b = \text{CO}_2$  in mm per liter of blood,  $(\text{CO}_2)_{bl} = \text{CO}_2$  in mm per liter of blood-saline-indicator mixture, and  $(\text{CO}_2)_l = \text{CO}_2$  in mm per liter of saline-indicator solution.

From these data (*i.e.*,  $\text{pH}_s$ ,  $(\text{CO}_2)_b$ , and volume of cells,  $V_c$ ), the bicarbonate concentration of serum,  $(\text{BHCO}_3)_s$ , and the  $\text{CO}_2$  tension of the blood,  $p\text{CO}_2$ , may be calculated by means of the nomogram given in Paper II of this series.

#### EXPERIMENTAL

A large series of analyses was made to compare the accuracy of the technique described here with that of standard methods with larger quantities of blood. As would be anticipated, the determination of  $\text{pH}_s$  and of percentage of cells showed no differences in degree of accuracy. As the following data show, the determination of  $\text{CO}_2$  by the micromethod is less accurate than by the standard 1 cc. method.

In order to determine the maximum accuracy of  $\text{CO}_2$  determinations on standard  $\text{Na}_2\text{CO}_3$  solutions, twelve standard solutions of  $\text{Na}_2\text{CO}_3$ , varying in concentration from 10 to 40 mm per liter, were

analyzed by both methods. The average difference between the amount of  $\text{CO}_2$  found by the standard method and that found by the micromethod was  $\pm 0.14$  mm per liter.

In order to compare  $\text{CO}_2$  analysis of blood by the standard 1 cc. method and by the micromethod, sixteen different samples of blood were analyzed by both methods. The average difference of the determinations by the two methods was found to be  $\pm 0.24$  mm per liter.

TABLE III

*Comparison of Microtechnique with Standard Technique with 1 Cc. Samples of Whole Blood*

Experiment No.	Sample No.	$V_c$		$\text{pH}_s$ at $38^\circ$		$(\text{CO}_2)_b$	
		Micro	Macro	Micro	Macro	Micro	Macro
1	1	0 58	0.58	7.29	7.27	20.32	20.35
	2	0.58	0 58	7.27	7.27	20.53	20.75
	3	0 59				20.74	20.54
	4	0 59				20.42	20.34
Average.....		0 585	0 58			20 50	20.49
2	1	0 46	0.46	7.23	7.25	20.45	
	2	0 465	0.46	7.21	7.26	22.29	20.99
	3	0 465				20.75	21.15
	4	0 47				20.15	21.07
Average.....		0 465	0.46			20.91	21 07
3	1	0 42	0 42	7.32	7.32	18.28	18.06
	2	0 42	0.428	7.31	7.31	17.94	
	3	0.43				18.02	18.02
	4	0.43				18.15	
Average.....		0.425	0.42			18.10	18.04

In Table III are given representative analyses of three blood samples which illustrate the comparative accuracy of quadruplicate determinations on the same blood and the differences between the microtechnique and the standard technique.

The method described here is for the analysis of cutaneous blood obtained from the finger. As has been shown by Lundsgaard and Möller (3), such blood may be regarded as closely approximating arterial blood in composition.

*Effect of Standing*—It has been found that it is inadvisable to let the blood samples stand in the funnels under oil for more than 15 minutes, since appreciable changes in pH will be found, usually toward the acid side. However, if the blood is diluted in the pipette and centrifuged immediately or within 30 minutes, no detectable change in pH occurs within 3 hours. When the pipettes are allowed to stand longer than 30 minutes, a measurable change in pH occurs. On the other hand, no detectable change in CO<sub>2</sub> content is found even after standing 48 hours in the ice box at about 3°.

*Errors and Limitation of the Method*—By the micromethod the percentage of cells may be determined with an accuracy of  $\pm 1.0$  per cent, pH with an accuracy of  $\pm 0.02$  pH, and total CO<sub>2</sub> content with an accuracy of  $\pm 0.25$  mm (about 1 per cent). This degree of accuracy can be obtained consistently with reasonable care. There is considerable improvement in technique with practise. This is true both in filling the pipettes accurately and in running the CO<sub>2</sub> analyses. Extreme care is essential in filling the pipettes accurately, since any error in this operation is magnified 20-fold in the final result.

In the case of the CO<sub>2</sub> analyses, the criterion of accuracy set up has been that two determinations must check within 0.50 mm of CO<sub>2</sub> in order to be acceptable. To meet this standard it is best to fill four pipettes from each sample. With four available, it is very seldom that two will fail to meet the above criterion of agreement.

#### SUMMARY

With a specially designed pipette, a method has been devised for determining the acid-base balance of the blood on 0.1 cc. samples of finger blood.

Percentage of cells, pH of the serum, and total CO<sub>2</sub> content are determined on the same 0.1 cc. sample.

The accuracy of the determinations is for cells, 1.0 per cent; for pH, 0.02 pH; and for total CO<sub>2</sub> content,  $\pm 1.0$  per cent, or 0.24 mm.

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As may be seen, Equations 1 and 2 are too complex to be useful in making numerous routine calculations because of the time required and the possibility of error. Hence a nomogram (Fig. 1) was constructed to carry out these computations. It is con-

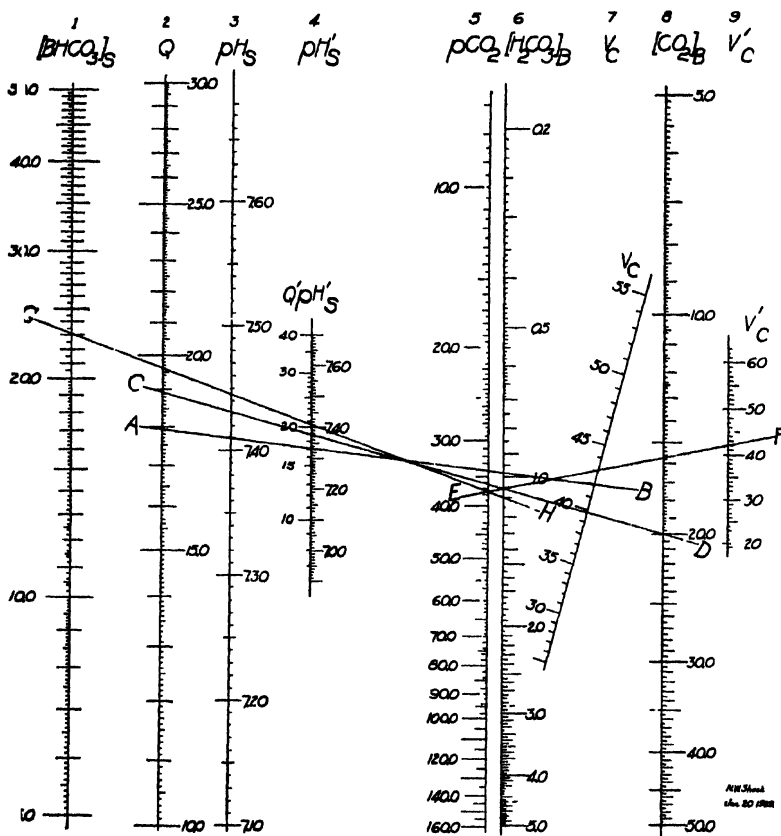


FIG. 1. Nomogram for blood calculations.  $(\text{BHC0}_3)_s$ ,  $(\text{H}_2\text{CO}_3)_b$ , and  $(\text{CO}_2)_b$  are expressed in mm per liter;  $p\text{CO}_2$  in mm. of mercury.

structed with intermediate scales in such a way that values of  $(\text{H}_2\text{CO}_3)_b$  and  $p\text{CO}_2$  may be determined, as well as  $(\text{BHC0}_3)_s$ . In addition to solving Equations 1 and 2, the nomogram may be used to solve the Henderson-Hasselbalch equation in its various forms for any of its variables.

$$pH_s = pK' + \log ((B\text{HCO}_3)_s / (H_2\text{CO}_3)_s) \quad (3)$$

$$pH = pK' + \log \frac{(CO_2)_b - 0.0591 pCO_2}{0.0591 pCO_2} \quad (4)$$

$$pCO_2 = 0.0591 ((CO_2)_b / (10^{(pK' - pH)} + 1)) \quad (5)$$

It should be noted that the nomogram as presented is useful for serum and for arterial and cutaneous blood.

### Use of Nomogram

*I. Calculation of  $(H_2CO_3)_b$ ,  $pCO_2$ , and  $(B\text{HCO}_3)_s$  from  $V_c$ ,  $pH_s$ , and  $(CO_2)_b$ —*Let  $V_c = 0.42$ ,  $pH_s = 7.41$ , and  $(CO_2)_b = 20.0$  mm per liter.

*Step 1*—Lay the straight edge on the chart (Fig. 1) so that its edge passes through the value for  $V_c$  on Scale 7 and through the value for  $pH_s$  on Scale 3. (Line  $AB$  of example, through  $V_c = 0.42$  and  $pH_s = 7.41$ .) Read the value of the point of intersection of this line and Scale 2 and record as  $Q$  (17.9 in example).

*Step 2*—Lay the straight edge so that its edge passes through the value of  $(Q + 1)$  on Scale 2, obtained by adding 1.0 to whatever value of  $Q$  was found above in Step 1, and the experimental value of  $(CO_2)_b$  on Scale 8. (Line  $CD$  of example through  $(Q + 1) = 18.9$  and  $(CO_2)_b = 20.0$  mm per liter.) Read the value of the point of intersection of this line and Scale 6 and record as  $(H_2CO_3)_b$  (1.05 in example).

*Step 3*—Without lifting the pencil from the intersection point of line  $CD$  and Scale 6 as obtained in Step 2, swing the straight edge around this point as a pivot until its edge passes through the  $V'_c$  scale (Scale 9) at the experimental value. Note that the same value is used here as in Step 1, but that the value is read on a different scale. (Line  $EF$  of example through  $(H_2CO_3)_b = 1.05$  and  $V'_c = 0.42$ .) Read the value of the point of intersection of this line and Scale 5 and record as  $pCO_2$  in mm. of mercury (37.5 mm. of Hg in example).

*Step 4*—Holding the  $pCO_2$  value just determined as a pivot, swing the straight edge around so that its edge intersects the  $pH'_s$  scale (Scale 4) in the proper value (7.41) and read off the  $(B\text{HCO}_3)_s$  at the intersection on Scale 1. (Line  $GH$  of example through  $pCO_2 = 37.6$  and  $pH'_s = 7.41$  of example, intersecting

( $\text{BHCO}_3$ ), scale at 23.2.) Note again that the same value is used for pH, here as was used in Step 1, but that it is read on a different scale.

*II. Calculation of pH, from  $p\text{CO}_2$  and  $(\text{CO}_2)_s$ .* Step 1—Lay the straight edge on Fig. 1 so that one edge passes through the proper value on the  $p\text{CO}_2$  scale (Scale 5) and also through the  $(\text{CO}_2)_s$  value on Scale 1. (Note that this scale is marked  $(\text{BHCO}_3)_s$ .) Read the point of intersection of this line on the  $Q'$  scale (Scale 4, left side).

Step 2—Subtract 1 from this value of  $Q$ , and opposite this value ( $Q - 1$ ) read the proper pH on the pH' scale (Scale 4, right side).

*III. Calculation of pH, from  $p\text{CO}_2$  and  $(\text{BHCO}_3)_s$ .*—Lay the straight edge on Fig. 1 so that one edge passes through the proper value on the  $p\text{CO}_2$  scale (Scale 5) and also through the  $(\text{BHCO}_3)_s$  value on Scale 1. Read the point of intersection of this line with the pH' scale (Scale 4).

*IV. Calculation of  $p\text{CO}_2$  from pH, and  $(\text{CO}_2)_s$ .* Step 1—Opposite the pH, on Scale 4, read the value of  $Q$ .

Step 2—Add 1.0 to this value of  $Q$ . Lay the straight edge on Fig. 1 so that one edge passes through this value ( $Q + 1$ ) on the  $Q'$  scale and through the proper value of  $(\text{CO}_2)_s$  on Scale 1, which is marked  $(\text{BHCO}_3)_s$ , and read the value of  $p\text{CO}_2$  at the point of intersection of the line of the straight edge and Scale 5.

*V. Calculation of  $p\text{CO}_2$  from pH, and  $(\text{BHCO}_3)_s$ .*—A straight line drawn through given points on Scales 1 and 4 cuts Scale 5 in the proper  $p\text{CO}_2$  value.

*VI. Calculation of  $(\text{BHCO}_3)_s$  from  $(\text{CO}_2)_s$  and pH.* Step 1—From the values of  $(\text{CO}_2)_s$  and pH, calculate  $p\text{CO}_2$  as in (IV) above.

Step 2—Through this value of  $p\text{CO}_2$  on Scale 5 and pH, on Scale 4 a line may be drawn intersecting the  $(\text{BHCO}_3)_s$  scale (Scale 1) in the desired value.

*VII. Calculation of  $(\text{BHCO}_3)_s$  from  $(\text{CO}_2)_s$  and  $p\text{CO}_2$ .* Step 1—pH, may be calculated from  $(\text{CO}_2)_s$  and  $p\text{CO}_2$  as in (II) above.

Step 2—A line through this pH, value on Scale 5 intersects Scale 1 at the proper  $(\text{BHCO}_3)_s$  value.

### Construction of Nomogram

Since the theory and method of constructing nomograms have been discussed previously in the literature, and may be found

in standard texts, details will not be presented in the following construction (1, 3, 4, 7).

Owing to the complexity of Equations 1 and 2, the nomogram for their solution is constructed in steps. In this way it is also possible to give intermediate values if they are desired.<sup>1</sup>

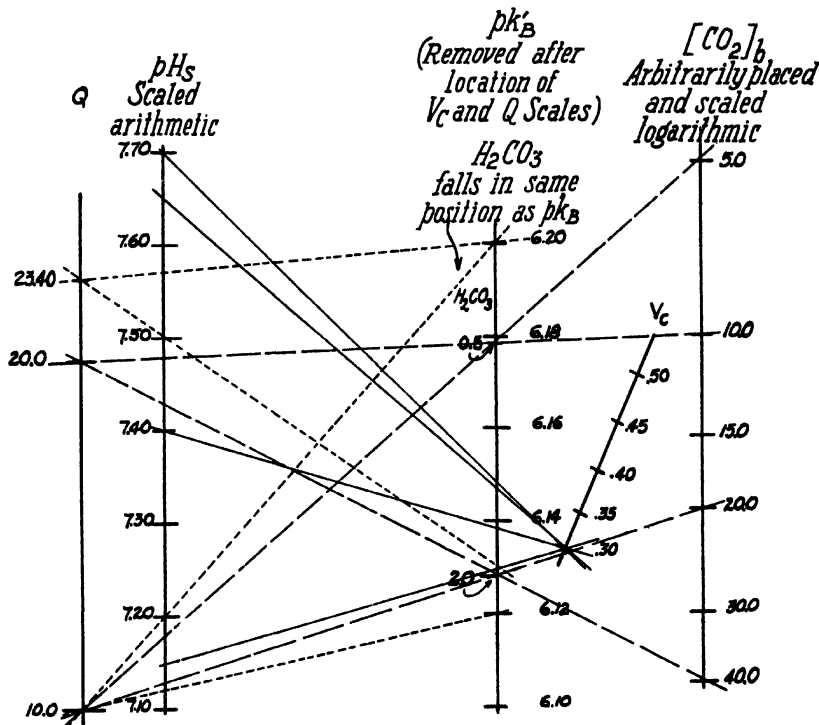


FIG. 2. Method for constructing the nomogram. The solid line represents derivation of  $V_c$  scale; the dotted line, derivation of  $Q$  scale; the dashed line, derivation of  $H_2CO_3$  scale. All other scales are located and calibrated in a similar manner.

**Construction of  $V_c$  Scale**—This scale is determined by empirical methods and is not theoretically exact, although the error is slight (less than 0.1 per cent).

<sup>1</sup> The tables of data illustrating the calculations involved in the preparation of the nomogram may be obtained by applying to the authors.

The  $pK'_b$  scale and the  $pH_b$  scale are first laid off arithmetically as in Fig. 2. The points on the  $V_c$  scale are determined by the intersection of four lines drawn as directed later. If a theoretically exact solution were possible by graphic methods, these four lines would meet in a point. As a matter of fact this is not the case, although the deviation is so slight that it is negligible. The given  $V_c$  point is taken as that nearest the point of intersection of the four lines drawn.

This variation is practically zero for the  $V_c = 0.45$  point and increases slightly in each direction.

An equation relating  $pK'_b$ ,  $V_c$ , and  $pH_b$  may be derived in the following form:

$$pK'_b = \log \frac{1 - 0.14V_c}{1 + V_c (1.23 - 0.223pH_b)} + 6.10$$

From this equation, values of  $pH_b$  and  $pK'_b$  may be obtained for constant  $V_c$  values. A table is then constructed for all useful values of  $V_c$ . The proper lines are then drawn (see Fig. 2) and the  $V_c$  points obtained.

*Construction of Q Scale*—An equation relating  $pH_b$ ,  $pK'_b$ , and  $Q$  may be derived from the modified Henderson-Hasselbalch equation for whole blood:

$$pH_b - pK'_b = \log \frac{(\text{CO}_2)_b - (\text{H}_2\text{CO}_3)_b}{(\text{H}_2\text{CO}_3)_b}$$

Let

$$((\text{CO}_2)_b - (\text{H}_2\text{CO}_3)_b) / (\text{H}_2\text{CO}_3)_b = Q$$

Then  $pH_b - pK'_b = \log Q$ .

This equation is readily plotted by nomographic methods from the  $pH_b$  and  $pK'_b$  scales already drawn (Fig. 2). The position of the scale with respect to the  $pH_b$  and  $pK'_b$  scales may be readily determined from the intersection of two lines giving the same  $\log Q$ , or  $Q$ , values. Intermediate values are obtained either by calibrating the scale for  $Q$  logarithmically between the determined points, or by solving for given values of  $Q$ , and finding intersection points on the  $Q$  scale. After the  $Q$  scale has been thus established and calibrated, the  $pK'_b$  scale may be removed, since it is no longer needed.

*Construction of  $(\text{H}_2\text{CO}_3)_s$  Scale*—Since

$$Q = ((\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s) / (\text{H}_2\text{CO}_3)_s \\ (\text{H}_2\text{CO}_3)_s = (\text{CO}_2)_s / (Q + 1)$$

This is also an equation that is easily solved nomographically. The  $(\text{CO}_2)_s$  scale (Fig. 1, Scale 8) is arbitrarily drawn and calibrated logarithmically. The  $(\text{H}_2\text{CO}_3)_s$  scale (Fig. 1, Scale 6) may be located in the same manner as the  $Q$  scale. Intermediate points may be determined logarithmically or by solution of the equations.

*Construction of  $p\text{CO}_2$  Scale*—It may be shown that  $p\text{CO}_2 = (\text{H}_2\text{CO}_3)_s / 0.0301 (1 - 0.14V_c)$ . This may be solved if the  $V_c$  scale is calibrated according to  $\log (0.0301 (1 - 0.14V_c))$ . Scale 9, Fig. 1, designated as  $V'_c$ , is calibrated in this way. The  $p\text{CO}_2$  scale may be located as before from  $V'_c$  (Scale 9) and  $(\text{H}_2\text{CO}_3)_s$  (Scale 6). Intermediate points may be obtained graphically or by calibrating the scale logarithmically.

*Construction of  $\text{pH}'_s$  Scale*—From the Henderson-Hasselbalch equation,  $(\text{BHCO}_3)_s = 10^{(\text{pH}_s - 6.10)} \times 0.0301 p\text{CO}_2$ .

The  $(\text{BHCO}_3)_s$  scale (Scale 1) is first laid off and calibrated logarithmically. The  $\text{pH}'_s$  scale is then located from this scale and the  $p\text{CO}_2$  scale. Since this scale will be arithmetical, it is quite simple to calibrate by dividing the distance between 7.10 and 7.40 into 30 equivalent parts, and by using this unit to extend the calibration above 7.40 and below 7.10.

*Construction of  $Q'$  Scale*—This scale is laid off logarithmically on the other side of the  $\text{pH}'_s$  scale from the Henderson-Hasselbalch equation,

$$\text{pH}_s = 6.10 + \log \frac{(\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s}{(\text{H}_2\text{CO}_3)_s}$$

but

$$((\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s) / (\text{H}_2\text{CO}_3)_s = Q'$$

therefore,  $\text{pH}_s - 6.10 = \log Q'$ . In other words, for any given  $\text{pH}$  value, a corresponding  $Q'$  value may be assigned, and *vice versa*.

#### DISCUSSION

Peters, Bulger, and Eisenman (5) have published a chart for the estimation of the  $\text{CO}_2$  content of the plasma from that of whole

blood, but it is limited in application to oxygenated blood with known  $\text{CO}_2$  tension.

Van Slyke and Sendroy (8) have published a nomogram for the estimation of a factor  $f$ , by which whole blood  $\text{CO}_2$  contents are multiplied to give the  $\text{CO}_2$  content of serum. This factor depends in part on  $\text{pH}_s$ , but principally on cell volume (or  $\text{O}_2$  capacity). Corrections are introduced so that the nomogram may be used for bloods of varying degrees of  $\text{O}_2$  saturation.

In addition, Van Slyke and Sendroy (8) have published a nomogram for the solution of the Henderson-Hasselbalch equation. The following calculations may be made by means of their nomograms.

$\text{pH}_s$	from $\text{pCO}_2$ and $(\text{CO}_2)_s$ or $\text{pCO}_2$ and $(\text{BHC}\text{O}_3)_s$
$\text{pCO}_2$	" $\text{pH}_s$ and $(\text{CO}_2)_s$ or $\text{pH}_s$ and $(\text{BHC}\text{O}_3)_s$
$(\text{BHC}\text{O}_3)_s$	" $(\text{CO}_2)_s$ and $\text{pH}_s$ or $(\text{CO}_2)_s$ and $\text{pCO}_2$

They have pointed out that for the calculations involving only  $\text{pCO}_2$ ,  $\text{pH}_s$ , and  $(\text{BHC}\text{O}_3)_s$ , the nomogram is theoretically exact. For calculations in which  $(\text{CO}_2)_s$  is involved the accuracy of the chart is approximate. The degree of approximation is so close, however, for the range of values found in either normal or pathological blood, that the error of calculation is well within that of the experimental determinations involved.

By the use of the intermediate value  $Q$ , the nomogram here presented is constructed theoretically exact for all calculations with the Henderson-Hasselbalch equation. This may be seen from the following considerations,  $\text{pH}_s - 6.10 = \log Q = \log ((\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s)/(\text{H}_2\text{CO}_3)_s$ .

For every  $\text{pH}_s$  value there is a corresponding  $Q$  value; hence in any equation involving  $\text{pH}_s$ , a transfer may be made in terms of  $Q$ , and the following equation solved for the required variable by subtracting or adding to the value of  $Q$  obtained. (See directions under "Use of nomogram," (II) and (IV).)

$$Q = ((\text{CO}_2)_s - (\text{H}_2\text{CO}_3)_s)/(\text{H}_2\text{CO}_3)_s$$

$$(\text{H}_2\text{CO}_3)_s = (\text{CO}_2)_s/(Q + 1)$$

$$(\text{CO}_2)_s = (\text{H}_2\text{CO}_3)_s (Q + 1)$$

The precision of the graphic, as well as the algebraic, application of the Henderson-Hasselbalch equation depends on the accuracy of the constants and  $\text{pK}'$ .

For the estimation of  $(\text{CO}_2)_a$  from  $(\text{CO}_2)_b$ , the nomogram of Van Slyke and Sendroy has an advantage over the present one, in that the former may be used for bloods with any degree of  $\text{O}_2$  unsaturation, while the latter is designed for use with arterial or capillary blood alone.

In order to check the accuracy of calculations of  $(\text{CO}_2)_a$  by means of the nomogram, data from the literature, collected by Van Slyke and Sendroy (8), were used. For details as to the source of these data reference may be made to their paper.

Values of  $(\text{CO}_2)_a$  calculated by our nomogram from  $(\text{CO}_2)_b$ ,  $V_c$ , and pH, are on the average 0.05 mm ( $\pm 0.002$ ) higher than those found by direct analysis of the true serum in forty-six analyses. The average deviation is 0.32 mm.

From these calculations it is shown that the  $(\text{CO}_2)_a$  may be calculated from pH,  $V_c$ , and  $(\text{CO}_2)_b$  with an error rarely exceeding 0.8 mm and in the majority of cases under 0.4 mm (average 0.32 mm).

This accuracy is of the same order of magnitude as that of the nomogram of Van Slyke and Sendroy for the estimation of  $(\text{CO}_2)_a$  from  $(\text{CO}_2)_b$ .

#### SUMMARY

A nomogram has been constructed with theoretically exact scales for the solution of the Henderson-Hasselbalch equation, in all its forms.

Scales have also been constructed to permit the estimation of  $(\text{H}_2\text{CO}_3)_b$ ,  $p\text{CO}_2$ , and  $(\text{BHCO}_3)_a$  from  $V_c$ , pH, and  $(\text{CO}_2)_b$ .

The  $(\text{BHCO}_3)_a$  may be estimated with an accuracy of 0.4 mm.

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## STUDIES OF THE ACID-BASE BALANCE OF THE BLOOD\*

### III. VARIATION IN THE ACID-BASE BALANCE OF THE BLOOD IN NORMAL INDIVIDUALS

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The aim of the present paper is: (a) to determine the range of variation in the acid-base balance of normal individuals; (b) to determine the amount of variation within a given individual from day to day with respect to the acid-base balance of his blood; and (c) to determine the fluctuation in the acid-base balance of the blood in normal individuals throughout the day.

#### *Methods*

The acid-base determinations were made by the microtechnique previously reported (13). From the percentage of red blood cells ( $V_c$ ), pH, and total  $\text{CO}_2$  content of the whole blood,  $(\text{CO}_2)_b$ , determined experimentally, the  $p\text{CO}_2$  and the bicarbonate content of the serum,  $(\text{BHCO}_3)_s$ , were calculated with the nomographic chart presented in Paper II of this series (8).

#### EXPERIMENTAL

*Variation in Acid-Base Balance of Different Individuals*—Tables I and II present the results of blood analyses made on thirty-nine male and seventeen female freshmen in the University of Chicago. These figures are the means of as many determinations as could be conveniently made on each individual, varying from one to nine samples.

No two samples drawn on the same day were used in the aver-

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TABLE I  
*Acid-Base Balance of Blood in Normal Men*

	No. of observations	V <sub>o</sub>	pH <sub>s</sub> at 38°	(CO <sub>2</sub> ) <sub>b</sub> <i>mm per l.</i>	pCO <sub>2</sub> <i>mm. Hg</i>	(BHCO <sub>3</sub> ) <sub>s</sub> <i>mm per l.</i>
Aag	1	0.56	7.42	19.64	38.4	24.2
Bat	1	0.445	7.43	23.86	43.2	27.7
Ben	1	0.47	7.45	22.78	40.2	27.0
Bre	1	0.41	7.41	24.43	45.3	27.7
Fer	1	0.43	7.40	21.40	41.1	24.7
Goe	1	0.50	7.43	22.50	41.8	26.9
Joh	1	0.51	7.37	21.69	45.7	25.6
Jor	1	0.47	7.36	23.52	49.7	27.2
Mal	1	0.50	7.40	22.68	47.4	28.5
Neu	1	0.475	7.45	22.91	40.5	27.2
Scol	1	0.455	7.38	21.86	44.0	25.2
Scot	1	0.44	7.43	22.81	41.3	26.5
Sem	1	0.43	7.41	19.18	36.0	22.2
Tut	1	0.455	7.45	22.35	39.1	26.3
Ande	2	0.46	7.43	22.02	40.3	25.9
Alb	2	0.455	7.38	21.35	43.5	24.6
Fis	2	0.48	7.38	22.30	45.6	26.0
Feth	2	0.50	7.42	22.12	42.0	26.4
Fris	2	0.46	7.38	23.87	47.9	27.5
Keel	2	0.47	7.34	24.14	53.9	27.9
Mark	2	0.48	7.40	23.90	46.8	28.1
Sch	2	0.47	7.39	21.06	42.2	24.5
Sev	2	0.47	7.39	20.86	42.0	24.3
Whi	2	0.48	7.41	20.04	38.9	23.6
Bach	3	0.48	7.38	20.58	42.3	24.0
El	3	0.49	7.39	21.91	44.1	25.8
Ma	3	0.485	7.40	21.69	42.7	25.5
Rob	3	0.50	7.37	23.14	48.5	27.3
Ask	4	0.51	7.41	23.16	45.4	27.7
Em	4	0.48	7.35	22.52	50.4	26.7
Spa	4	0.49	7.41	21.82	42.4	25.8
Tur	4	0.49	7.39	22.52	45.5	26.6
Andd	5	0.473	7.44	22.17	40.2	26.2
Scota	5	0.474	7.40	21.73	42.7	25.5
Spi	5	0.49	7.38	22.75	46.6	26.8
Too	5	0.471	7.41	22.96	43.6	27.0
Jen	6	0.475	7.36	24.23	51.2	28.0
Mel	7	0.50	7.37	23.08	46.1	27.5
Dar	9	0.449	7.40	22.25	42.7	25.8
Mean.....		0.4752	7.399	22.302	43.88	26.20
Standard deviation of distribution.....		0.029	0.064	1.204	3.697	1.452
Standard error of mean.....		0.0046	0.0102	0.193	0.592	0.2325
Probable error of mean		0.0031	0.0069	0.130	0.399	0.157

ages of Tables I and II. Samples ~~were~~ drawn at all hours of the day with no control of diet or activity of the individual. Hence the variation found may be a function of these factors, rather than any actual difference in the bloods of the normal humans. However, evidence to be presented later indicates that the variation found is not entirely a function of diet and activity.

TABLE II  
*Acid-Base Balance of Blood in Normal Women.*

	No. of observations	V <sub>c</sub>	pH <sub>s</sub>	(CO <sub>2</sub> ) <sub>s</sub> <i>mm per l.</i>	pCO <sub>2</sub> <i>mm. Hg</i>	(BHCO <sub>3</sub> ) <sub>s</sub> <i>mm per l.</i>
Chi	1	0.425	7.39	22.73	44.4	26.0
Bad	1	0.47	7.41	20.42	39.0	24.0
Fric	1	0.45	7.42	20.94	38.8	24.5
Gon	1	0.375	7.38	22.34	43.5	24.8
Good	1	0.40	7.38	18.80	36.9	21.2
Gren	1	0.39	7.38	22.01	39.2	22.5
Gre	1	0.405	7.45	22.15	37.6	25.3
Holm	1	0.42	7.38	21.00	41.8	23.9
McK	1	0.37	7.42	21.51	38.3	24.2
Scha	1	0.39	7.45	25.00	42.4	28.5
Sho	1	0.41	7.43	21.92	38.9	25.1
Ada	2	0.45	7.43	23.36	43.0	27.3
Bail	2	0.428	7.42	22.88	42.0	26.4
Hala	2	0.37	7.40	20.94	38.9	23.4
Rog	2	0.40	7.43	22.59	40.1	25.8
Schm	2	0.38	7.44	21.92	37.6	24.7
Fisc	3	0.44	7.44	21.77	38.7	25.3
Mean.....		0.410	7.415	21.899	40.06	24.88
Standard deviation of distribution.....		0.029	0.025	1.359	2.261	1.680
Standard error of mean.....		0.0072	0.0059	0.330	0.548	0.407
Probable error of mean		0.0049	0.0040	0.223	0.370	0.275

Table III is a summary of the means and standard deviations of the distributions of the data of Tables II and III. In addition, the means and standard deviations for each variable were calculated, by using the total number of observations on each individual, instead of individual averages as in Tables I and II. These results are also given in Table III. As may be seen from Table

TABLE III  
*Sex Differences in Acid-Base Balance of Blood*

	No. of individuals tested*	$V_e$		pH <sub>s</sub>		$(CO_2)_s$		$pCO_2$		$(BHCO_3)_s$	
		Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$
Men.....	39	0.475 $\pm$ 0.003	0.029	7.399 $\pm$ 0.007	0.064	22.30 $\pm$ 0.13	1.204	43.88 $\pm$ 0.40	3.70	26.20 $\pm$ 0.16	1.45
Women.....	17	0.410 $\pm$ 0.005	0.029	7.415 $\pm$ 0.004	0.025	21.899 $\pm$ 0.223	1.359	40.06 $\pm$ 0.37	2.26	24.88 $\pm$ 0.27	1.68
Men and women.....	57	0.455 $\pm$ 0.004	0.041	7.402 $\pm$ 0.008	0.037	22.19 $\pm$ 0.11	1.245	42.86 $\pm$ 0.35	3.96	25.79 $\pm$ 0.11	1.26
Single observations.....	131	0.465 $\pm$ 0.002	0.038	7.399 $\pm$ 0.004	0.070	22.34 $\pm$ 0.08	1.391	43.68 $\pm$ 0.27	4.67	26.10 $\pm$ 0.10	1.69

$(CO_2)_s$  and  $(BHCO_3)_s$  are given in mm per liter of blood;  $pCO_2$  in mm. of mercury.

$\sigma_d$  = standard deviation of the distribution.

\* Each figure used for an individual is the mean of all observations made on that individual.

III, the women show a slightly increased variation in  $V_c$  and  $pH_s$ , while there is no significant difference in variation in the other measures. It is also noticeable that by using the individual observations the variability of all measures is increased.

Statistical tests applied to the difference between the mean values for men and women show that differences are significant in the case of  $V_c$ , and probably so in the case of  $pCO_2$  and  $(BHCO_3)_s$ , the men showing slightly higher values in all three measures. Table IV shows the results of these tests.

The graphic representation of these data is shown in Fig. 1, in which the  $pH_s$ ,  $(BHCO_3)_s$ , and  $pCO_2$  for each individual studied have been plotted on triaxial coordinates (9). Since triaxial cross

TABLE IV  
*Difference in Means for Men and Women*

	$V_c$	$pH_s$	$(CO_2)_b$	$pCO_2$	$(BHCO_3)_s$
Mean of men.....	0.475	7.399	22.302	43.88	26.20
" " women.....	0.410	7.415	21.899	40.06	24.88
Difference....	0.065	-0.016	0.403	3.82	1.32
Probable error of difference...	0.006	0.008	0.258	0.544	0.317
Difference/probable error of difference.....	10.83	2.00	1.56	7.02	4.16

$(CO_2)_b$  and  $(BHCO_3)_s$  are given in mm per liter of blood;  $pCO_2$  in mm. of mercury.

section paper is usually ruled with equal spacings between the lines and at a 60° angle, it is adapted to plotting data related by the equation,  $ax - by + cz = k$ . From the Henderson-Hasselbalch equation,  $pH = pK' + \log (BHCO_3/H_2CO_3)$  and  $pK' = pH - \log BHCO_3 + \log H_2CO_3$ . It is evident that the  $pH$ , the  $\log BHCO_3$ , and the  $\log H_2CO_3$  may be plotted arithmetically on the three axes of such paper. The advantage of this method of plotting the data is that constant  $H_2CO_3$  (or  $CO_2$  tension), constant  $pH$ , and constant  $BHCO_3$  lines are rectilinear and are inclined at equal angles to each other. Hence paths of displacement of the acid-base balance may be evaluated more readily in terms of both direction and extent. In addition, one may estimate the degree of participation of respiratory or metabolic factors in producing a given path of displace-

ment. Constant pH lines run vertically (north and south); constant  $\text{BHCO}_3$  lines run northwest and southeast; constant  $p\text{CO}_2$  lines run northeast and southwest. The small interior hexagon of solid lines represents the limits of normal variation in males.

When so plotted, the data of the male subjects (Fig. 1) fall preponderantly within an area bounded by pH limits of 7.35 and 7.45, by  $(\text{BHCO}_3)$ , limits of 23 and 30 mm per liter, and by  $p\text{CO}_2$  limits of 40 and 50 mm. of mercury. Of the thirty-nine individuals so studied, three had  $\text{CO}_2$  tensions higher than 50 mm. and

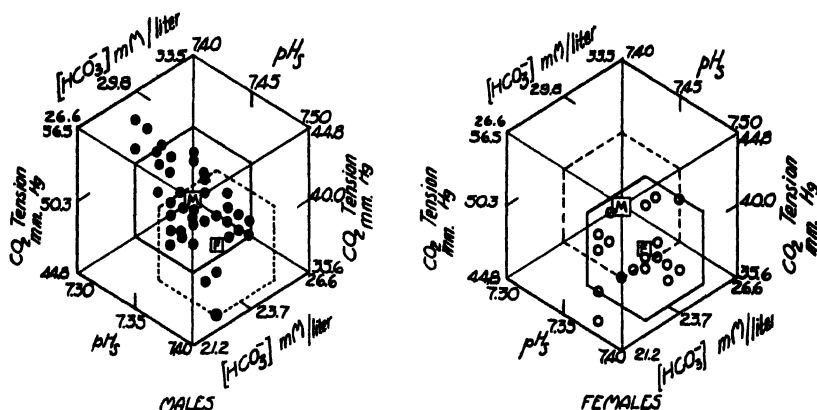


FIG. 1. Variation in acid-base balance among normal individuals. The solid black circles represent the values for men; the clear circles, those for women. The squares are the means of the observations of the values for M and F.

three had  $\text{CO}_2$  tensions lower than 40 mm. When the data of the seventeen female subjects were similarly plotted, the points failed to fall symmetrically within the limits found for the males. Although we recognize that our series of observations is small, nevertheless the data on the female subjects seem to be sufficiently different to justify the conclusion that the normal area of females differs slightly from that of males. Tentatively we have defined the normal area for women as limited by pH limits of 7.37 and 7.47, by  $(\text{BHCO}_3)$ , limits of 22 and 28 mm per liter, and by  $p\text{CO}_2$  limits of 46 and 36.5 mm. of mercury. The mean of the data for men is designated on the chart as a square containing the letter

$M$ , and represents  $\text{pH} = 7.40$ ,  $(\text{BHCO}_3)_s = 26.6$ , and  $\text{pCO}_2 = 44.8$ . The mean for the women is designated as  $F$  within a square, and represents  $\text{pH} = 7.42$ ,  $(\text{BHCO}_3)_s = 25.0$ , and  $\text{pCO}_2 = 41$ .

This apparent difference between the mean normal value of the acid-base balance of the blood in females and males is what one would expect if the  $\text{CO}_2$  tension of the blood is 4 mm. less in females than in males. Haldane (7) has reported that the  $\text{CO}_2$  tension of the alveolar air of women is 3 mm. of Hg less than that of men. This is consistent with our results on finger blood. The slightly lower  $(\text{BHCO}_3)_s$  and higher  $\text{pH}$ , are simply the result of reducing the  $\text{CO}_2$  tension of the blood, and the two points for the means of  $M$  and  $F$  consequently fall approximately on the  $\text{CO}_2$  absorption curve of normal blood.

Examination of the data in which more than one determination of the acid-base balance had been made on the same individual showed that within the limits of normal variation the second determination could not be predicted with any degree of accuracy from the first, except in the case of cell volume, which remained relatively constant for a given individual.

*Variation in Acid-Base Balance of an Individual from Day to Day*—In order to investigate the variation in acid-base balance in a given individual from day to day, the following experiment was carried out. Eight men and seven women, graduate students, came to the laboratory daily for 30 to 34 days. Each subject came at the same hour of the morning, 6 days a week, without breakfast. Before the blood samples were drawn, each subject spent approximately half an hour working on a series of mental tests, so that the effects of muscular activity were probably minimized.

Table V is a summary of the data, giving the mean value, its probable error, and the standard deviation of the distribution of each variable for all fifteen subjects. Normal data from six subjects used in other later experiments are also included. Comparison of values in Table V with those in Table III shows that, in some individuals, the variation from day to day is as great as the variation between individuals, while in others the variation is significantly less. Statistical tests of significance show that the



TABLE V  
Variation in Acid-Base Balance from Day to Day in Normal Individuals

Sub- ject No.	No. of obser- vations	V <sub>c</sub>		pH <sub>a</sub>		(CO <sub>2</sub> ) <sub>b</sub>		pCO <sub>2</sub>		(BHCO <sub>3</sub> ) <sub>a</sub>	
		Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$	Arithmetical mean	$\sigma_d$

Men											
						$\frac{mM}{per\ l.}$	$\frac{mm. Hg}{}$	$\frac{mm. Hg}{}$	$\frac{mm. Hg}{}$	$\frac{mM}{per\ l.}$	$\frac{mM}{per\ l.}$
3	33	0.450 ± 0.004	0.030	7.366 ± 0.007	0.056	22.02 ± 0.121	1.03	45.83 ± 0.686	5.84	25.24 ± 0.171	1.46
4	34	0.434 ± 0.003	0.024	7.341 ± 0.008	0.065	21.70 ± 0.140	1.21	47.33 ± 0.570	4.93	24.89 ± 0.177	1.53
8	30	0.464 ± 0.002	0.018	7.404 ± 0.005	0.044	21.14 ± 0.078	0.63	40.93 ± 0.406	3.30	24.88 ± 0.115	0.93
9	31	0.465 ± 0.002	0.015	7.342 ± 0.009	0.071	22.01 ± 0.136	1.12	48.52 ± 0.569	4.70	25.51 ± 0.177	1.46
11	30	0.472 ± 0.002	0.019	7.346 ± 0.006	0.047	21.70 ± 0.137	1.11	47.48 ± 0.602	4.89	25.21 ± 0.183	1.49
12	30	0.461 ± 0.002	0.016	7.365 ± 0.007	0.054	22.22 ± 0.106	0.86	46.60 ± 0.642	5.21	25.81 ± 0.129	1.05
13	30	0.472 ± 0.002	0.018	7.326 ± 0.003	0.028	22.22 ± 0.134	1.09	50.92 ± 0.580	4.71	25.80 ± 0.144	1.17
14	28	0.507 ± 0.002	0.017	7.345 ± 0.004	0.030	22.25 ± 0.158	1.24	49.55 ± 0.614	4.82	26.31 ± 0.345	2.73
15	29	0.451 ± 0.003	0.018	7.372 ± 0.007	0.052	21.86 ± 0.159	1.27	45.00 ± 0.690	5.51	25.30 ± 0.197	1.57
17	22	0.465 ± 0.002	0.017	7.403 ± 0.009	0.063	21.75 ± 0.190	1.32	42.12 ± 0.438	3.04	25.45 ± 0.224	1.56
18	25	0.480 ± 0.001	0.007	7.387 ± 0.004	0.022	22.42 ± 0.025	0.19	45.13 ± 0.364	2.70	26.25 ± 0.135	1.00
19	14	0.457 ± 0.003	0.020	7.387 ± 0.009	0.054	21.76 ± 0.237	1.31	43.25 ± 0.476	2.64	25.18 ± 0.314	1.74
20	19	0.534 ± 0.004	0.028	7.362 ± 0.005	0.032	19.58 ± 0.140	0.91	42.61 ± 0.504	3.26	23.43 ± 0.111	0.71
21	20	0.490 ± 0.003	0.019	7.383 ± 0.014	0.062	22.46 ± 0.196	1.30	45.82 ± 0.695	4.61	26.38 ± 0.203	1.35

Women											
						$\frac{mM}{per\ l.}$	$\frac{mm. Hg}{}$	$\frac{mm. Hg}{}$	$\frac{mm. Hg}{}$	$\frac{mM}{per\ l.}$	$\frac{mM}{per\ l.}$
1	33	0.402 ± 0.004	0.031	7.358 ± 0.011	0.095	20.42 ± 0.135	1.15	42.40 ± 0.515	4.39	23.17 ± 0.236	2.01
2	33	0.389 ± 0.003	0.025	7.386 ± 0.012	0.099	21.94 ± 0.306	2.61	42.19 ± 0.584	4.97	24.80 ± 0.228	1.94
5	33	0.369 ± 0.004	0.031	7.381 ± 0.008	0.070	22.31 ± 0.217	1.85	43.26 ± 0.382	3.25	24.94 ± 0.244	2.08
6	33	0.357 ± 0.003	0.027	7.377 ± 0.007	0.059	21.06 ± 0.304	2.59	40.84 ± 0.628	5.35	23.34 ± 0.357	3.04
7	33	0.382 ± 0.002	0.021	7.358 ± 0.009	0.078	21.75 ± 0.241	2.05	44.22 ± 0.668	5.69	24.10 ± 0.228	1.94
10	28	0.374 ± 0.002	0.014	7.362 ± 0.006	0.046	22.01 ± 0.135	1.06	44.33 ± 0.500	3.92	24.44 ± 0.171	1.34
16	30	0.386 ± 0.002	0.013	7.395 ± 0.006	0.046	23.80 ± 0.206	1.67	45.32 ± 0.704	5.72	26.57 ± 0.126	1.02

$\sigma_d$  = standard deviation of the distribution.

differences between some individuals are too great to be attributed to chance.

Figs. 2 to 5 are sample time curves showing the variation in  $V_e$ ,  $pH_s$ , and  $(CO_2)_s$  found in given individuals from day to day. As may be seen, the fluctuation is much greater in the case of some individuals than in others. Subjects 5 and 13 show as much varia-

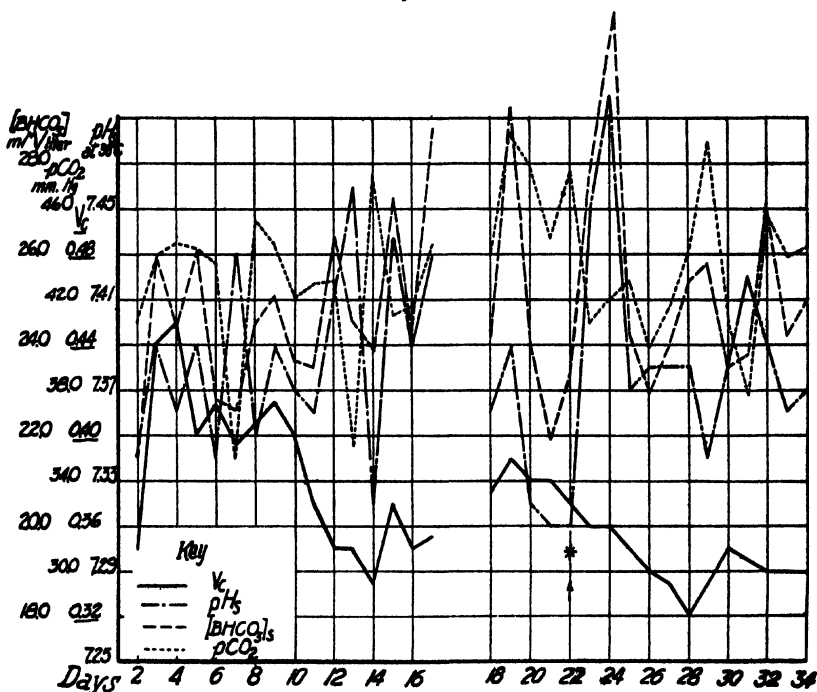


FIG. 2. Daily variation in acid-base balance of blood in Subject 5, female. There was a vacation period of 1 week between the 17th and 18th days of experiment. The asterisk indicates that 25 gm. of  $NaHCO_3$  were administered orally on the 22nd day. Maximum  $BHC0_3$  = 32.0 mm per liter. The determinations were made daily at 8.30 a.m.

tion in their blood as was found in the total range of normal individuals tested. Subjects 8 and 9 showed very little variation from day to day.

It is noticeable that these fluctuations are well outside the experimental error of the analytical method in the case of most subjects. That the fluctuations found are not the result of the tech-

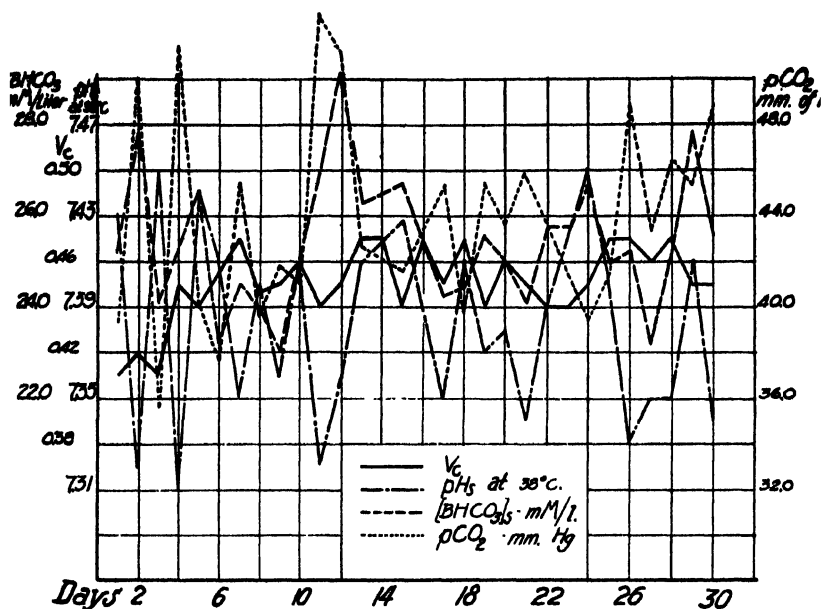


FIG. 3. Daily variation in acid-base balance of blood in Subject 15, male

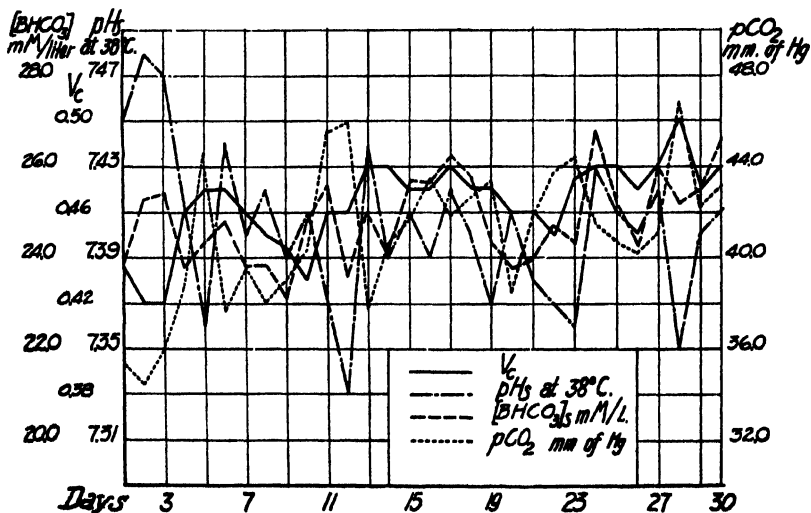


FIG. 4. Daily variation in acid-base balance of blood in Subject 8, male

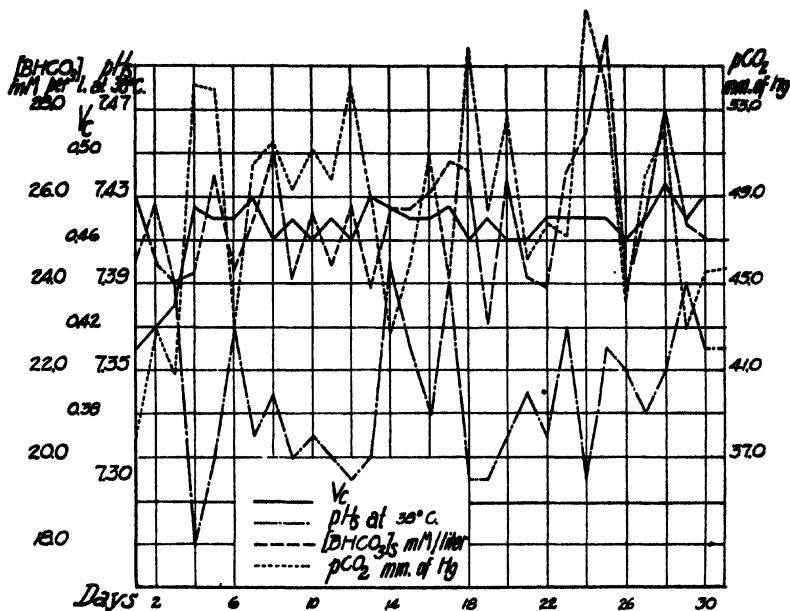


FIG. 5. Daily variation in acid-base balance of blood in Subject 9, male

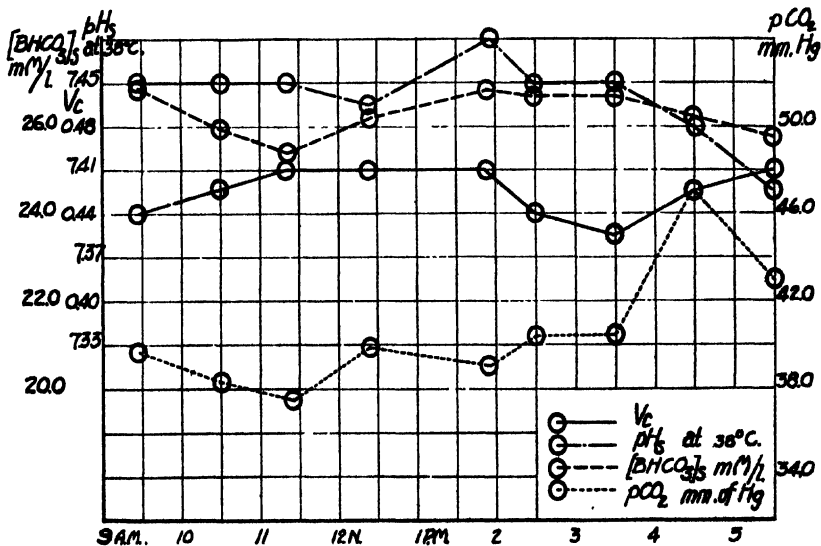


FIG. 6. Hourly variation in acid-base balance of blood in Subject 9, male, December 21, 1929.

nique is further borne out by the fact that, on a given day, the fluctuations of different individuals are not all in the same direction. Since the same solutions and technique were used for all subjects on a given day, one would expect the subjects to vary together, if the technique were the cause of the observed variation.

In addition, certain characteristics of the curves seem to indicate that the fluctuations found are true physiological phenomena. It has been found that frequently the data show general trends

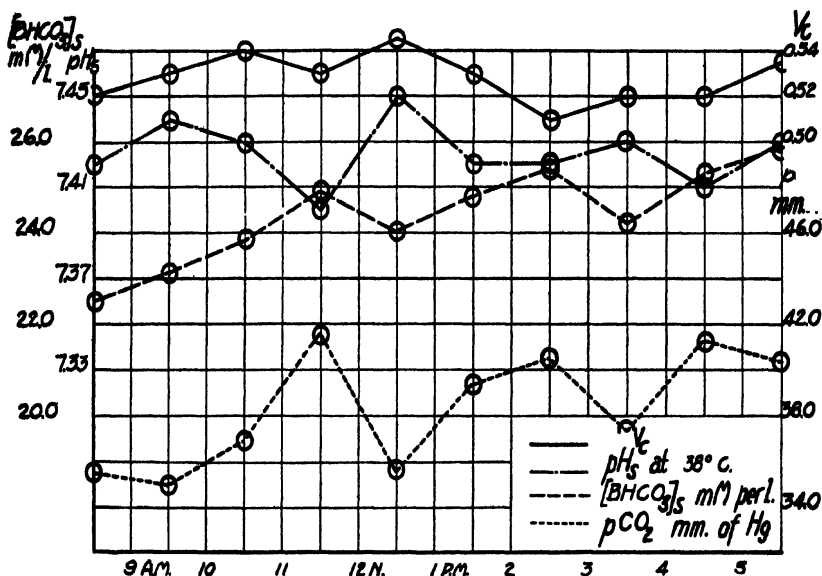


FIG. 7. Hourly variation in acid-base balance of blood in subject J. H. B., male, January 19, 1931.

over a period of several days. For instance, in Fig. 2, there is an average rise in pH extending from the 10th to the 16th day of the experiment, and a rise in (BHCO<sub>3</sub>)<sub>s</sub>. This was coincident with a drop in percentage cells, V<sub>c</sub>. Other such periods, or cycles, have been observed on the curves.

From these data it was apparent that the fluctuation of the acid-base balance of the blood within a given individual might be as great as the entire range of values found for normals.

*Hourly Variation in Acid-Base Balance of Blood of Normal*

*Individuals*—The next question arose as to how much fluctuation might be expected in the acid-base balance of a given individual throughout the course of a day. In this series, ten male subjects came to the laboratory at hourly intervals during a day from 8.30 a.m. to 6.30 p.m. for blood analyses. One subject served for five experiments; one subject served for three experiments; seven subjects served for two experiments each, making a total of twenty-two experiments. No control of diet or activity was made in

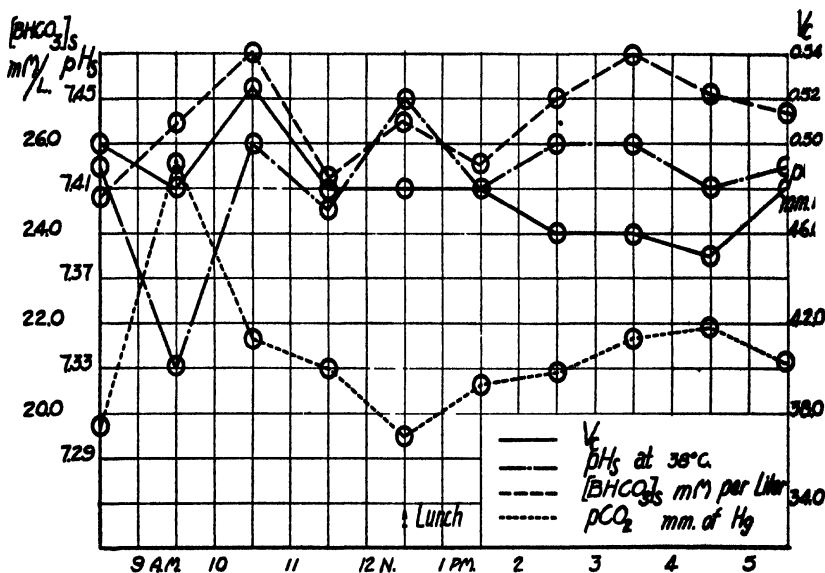


FIG. 8. Hourly variation in acid-base balance of blood in subject S. S., male, January 10, 1931.

these experiments. Some subjects stayed in the laboratory and studied during the entire day, while others came and went during the hourly intervals.

Figs. 6 to 9 present typical data graphically. The amount of variation found differs with individuals and with the same individual on different days. Fig. 8 shows results from a subject who always gave an erratic curve of this character, even though he was in the laboratory throughout the day.

When all the curves are examined, no general characteristics regarding changes in acid-base balance throughout the course of

the day seem apparent. While some curves show a slight drop in pH toward evening, others may show a slight rise. The "alkaline tide," reported by some observers, failed to appear consistently in these experiments, although present in some degree in Figs. 7, 8, and 9. On the other hand, a reverse effect, namely a drop in pH immediately after eating, was found in some experiments. This failure to find an alkaline tide may be due to the fact that blood samples were usually taken at 12.30 and 1.30 p.m., the subject eating his lunch in the interim. In some curves there appears to

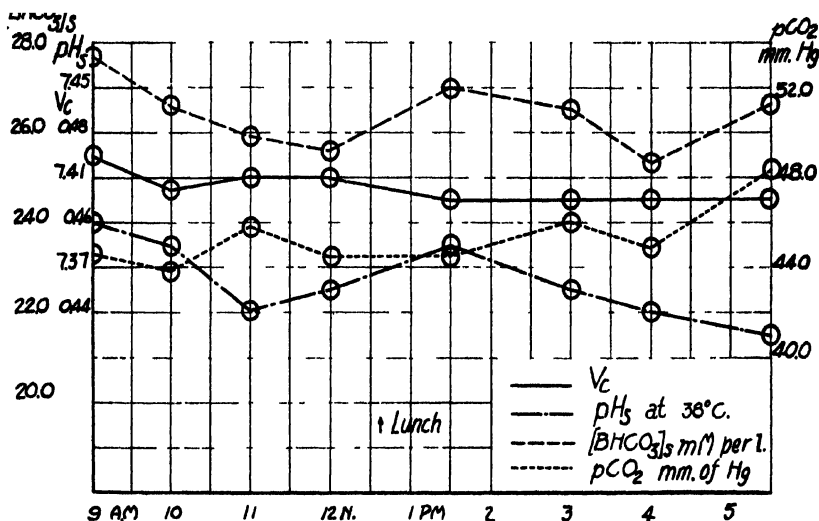


FIG. 9. Hourly variation in acid-base balance of blood in subject R. H. J., male, October 22, 1930.

be a slight rise in (BHCO<sub>3</sub>)<sub>s</sub> content during the afternoon (2.00 to 4.00 p.m.). Sometimes the curve drops again by 6.30 p.m., but not always.

It may be seen that, in general, the variation during the day is not as great as that observed from one day to another. The evidence lends support to the thesis that the variability shown in acid-base balance is a function of the given individual. It seems quite probable that such fluctuations may be due to differences in the reaction of the individual to various stimuli that tend to displace the normal acid-base balance.

## DISCUSSION

The range of normal variation in the acid-base condition of the blood has been studied by several investigators, including Cullen and Robinson (3), Myers and Booher (12), Bigwood (2), Marrack and Boone (11), Koehler (10), Austin and Cullen (1), and Earle and Cullen (4, 5).

The general results of these studies have indicated that the normal range is: for pH, 7.30 to 7.52; for total  $\text{CO}_2$  content of serum, 24 to 33 mm per liter; and for  $p\text{CO}_2$ , 34 to 62 mm. of mercury.

The results of this study are more in accordance with previous normal values found than with those reported by Earle and Cullen (5), whose values are higher in pH than those previously reported by about 0.06 to 0.07 pH.

Earle and Cullen (6) have also made a study of the diurnal variation in acid-base balance on ten subjects, drawing the blood by vena punctures. Five or six samples were drawn during the course of the day, the withdrawals beginning before breakfast, and concluding at about 10.00 p.m. The authors found an increase of from 0.01 to 0.07 pH in the course of the day, although the increase was interrupted by other fluctuations. Neither could an increase in pH and  $\text{CO}_2$  content be consistently demonstrated after meals in these subjects. In the case of all the curves presented, the pH curves were definitely higher, while the  $p\text{CO}_2$  curves were lower, than those found in the present study. (The lower  $p\text{CO}_2$  curves would necessarily follow from the higher pH values found, since  $p\text{CO}_2$  was calculated by the Henderson-Hasselbalch equation.) It is also true that some individuals from Earle and Cullen's series showed even greater fluctuations in acid-base balance than did the subjects in the present series.

The two studies are in agreement as to the general results, except for the fact that the general rise in pH reported by Earle and Cullen (6) could not be demonstrated in the present study. This may be due to the fact that our final sample was usually taken at 6.30 p.m., before the subject left the laboratory for dinner.

## SUMMARY

1. Normal variation in acid-base balance between males may be from pH 7.35 to 7.45;  $(\text{BHCO}_3)$ , from 23.0 to 30.0 mm per liter; and  $p\text{CO}_2$  from 40.0 to 50.0 mm. of mercury.



2. Normal variation in acid-base balance between females may be from pH 7.37 to 7.47; ( $\text{HCO}_3$ )<sub>s</sub> from 22.0 to 28.0 mm per liter; and  $p\text{CO}_2$  from 36.5 to 46.0 mm. of mercury.

3. Normal individuals differ significantly from one another in regard to the acid-base balance of their blood.

4. Some individuals show as much variation in acid-base balance from day to day as exists in the total number studied, while in other individuals there is little fluctuation.

5. No characteristic changes in acid-base balance during the course of the day were found in normal subjects. The amount of fluctuation in acid-base balance from hour to hour throughout the day seems to be a function of the individual, although the extent of hourly fluctuations may vary somewhat from day to day in the same individual.

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# THE REACTION OF NITROUS ACID WITH CYSTINE AND RELATED SULFUR-CONTAINING COMPOUNDS

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Van Slyke in 1911 (1) observed that the amount of nitrogen obtained from the reaction of cystine with nitrous acid in the amino nitrogen determination was from 7 to 8 per cent greater than the theoretical amount. Schmidt (2), in a critical study of the reaction between nitrous acid and amino acids, observed that when a solution of cystine was mixed with nitrous acid and barium chloride a copious precipitate of barium sulfate appeared within a few minutes, and suggested that, as the oxidation of the sulfur of the cystine molecule took place, the nitrous acid was reduced with the formation of nitrogen as one of the products. The chief evidence in support of the hypothesis advanced by Schmidt (2) was afforded by the data obtained with  $\beta$ ,  $\beta'$ -dithiodiglyceric acid (the desaminocystine of Neuberg and Ascher (3)). When this compound reacted with nitrous acid in the Van Slyke procedure, gas was evolved, and since this compound, if pure, contains no nitrogen, the implication was that any nitrogen thus obtained must have been derived from the reduction of nitrous acid. Two considerations, however, make this experiment less convincing than would have otherwise been the case; no figures are presented to indicate whether oxidation of the sulfur of  $\beta$ ,  $\beta'$ -dithiodiglyceric acid to sulfate occurred and the purity of the acid (prepared by the deamination of cystine) was not satisfactory.

Sanchez (4) has suggested a series of reactions to explain the oxidation of the sulfur of cystine by nitrous acid in which the formation of pyruvic acid as an intermediate product of the reaction is postulated; but no adequate experimental basis for the re-

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actions proposed is presented in the publications available to us. Since Clarke and Inouye (5) have reported that pyruvic acid yields a gas in the Van Slyke apparatus, it seemed possible that the "extra nitrogen" might be related to the presence of the pyruvic acid formed as a secondary product of the reaction of the deamination of cystine by nitrous acid.

In the work to be reported in this paper, attempts have been made to approach the problem of the reaction between cystine and nitrous acid by securing data along three related lines. When cystine is acted upon by nitrous acid, what degree of oxidation of the sulfur can be obtained? By the examination of a series of organic compounds in which the type of sulfur linkage varies, is it possible to establish any relationship between the oxidation of the sulfur to sulfate by nitrous acid and production of extra nitrogen in the Van Slyke reaction? What is the chemical nature of the gas produced when nitrous acid reacts in the Van Slyke apparatus with a nitrogen-free sulfur-containing compound of known purity whose sulfur is readily oxidized to sulfate by nitrous acid?

#### EXPERIMENTAL

Determinations of amino nitrogen were made according to the micromethod of Van Slyke in the modified reaction chamber described by Koch (6) at room temperature (approximately 25°). The nitrous acid was allowed to react with the compounds studied for periods of 3, 30, and 60 minutes, with continuous agitation of the deamination chamber during the periods of 3 and 30 minutes. In the longer periods, the deamination chamber was shaken during the first 3 and last 3 minutes of the reaction period.

In the study of the oxidation of the sulfur of the various compounds to sulfate by nitrous acid, barium nitrite was used as the source of the nitrous acid. As the oxidation of the organic sulfur to sulfate occurred the barium ion thus introduced served to precipitate the sulfate ion. All the oxidations were carried out in beakers covered with watch-glasses in an incubator at  $25^{\circ} \pm 1^{\circ}$ . In order to maintain this temperature from the beginning of the reaction, all the solutions used were kept in the incubator before combining the materials in the reaction mixture. The transfer, ignition, and weighing of the barium sulfate formed were accomplished with the usual precautions.

The solutions of the sulfur compounds to be studied were prepared so that 20 cc. of the solution would contain approximately 50 mg. of sulfur, except in the case of a few compounds where insolubility or other factors made this impracticable. To 20 cc. of the solution of the substance under investigation, 20 cc. of water and 30 cc. of 3.25 per cent hydrochloric acid (0.975 gm.) were added and the mixture was carefully stirred. Barium nitrite (30 cc. of a 5 per cent solution, *i.e.* 1.62 gm. of crystalline barium nitrite) was added, the solution was carefully mixed, and placed in the incubator. The time of the addition of the barium nitrite was taken as the beginning of the period of oxidation. Every precaution was taken to insure as uniform conditions as possible for the series of experiments.

In the reaction mixture as described above, the excess nitrous acid decomposes presumably to form nitric acid, nitric oxide, and water, according to the equation,  $3\text{HNO}_2 = \text{HNO}_3 + 2\text{NO} + \text{H}_2\text{O}$ . The problem presented itself as to whether the nitric acid thus formed might not be responsible for the oxidation of the sulfur in the experiments. Control experiments in which barium nitrate replaced an equivalent amount of nitrite in the reaction mixture demonstrated that nitric acid, when formed in the reaction from nitrous acid, was not responsible for the oxidation of the sulfur of the cystine.

Since in the procedure outlined above the beakers were covered with watch-glasses only, the oxides of nitrogen formed escaped rapidly. It seemed probable that with the decrease in the amount of the reagent thus resulting, the rate of oxidation of the sulfur might also decrease. If this were the case, addition of more barium nitrite, after the reaction had progressed, should result in the oxidation of a larger proportion of the organic sulfur to sulfate sulfur at the end of a given period than in a reaction mixture similarly treated except that no extra barium nitrite was added. In such an experiment, in which cystine served as the source of organic sulfur, extra barium nitrite (15 cc. of a 5 per cent solution), acid, and water were added at the end of the first 5.5 hours of a 24 hour period of oxidation so that the concentrations of the barium ion and the acid were essentially constant. 49.5 per cent of the cystine sulfur was recovered as sulfate sulfur in the series which had received no additional barium nitrite; while in

the series to which extra barium nitrite had been added, 53.1 per cent of the sulfur was obtained as sulfate. In similar experiments the use of a higher concentration of nitrite (10 M sodium nitrite) did not increase the formation of sulfate sulfur from the oxidation of cystine.

In Table I are presented the data of two typical series of experiments in which the rate of oxidation of the sulfur of cystine to sulfate by nitrous acid was studied over short time intervals. It will be noted that the reaction proceeded most rapidly in the first 15 minutes during which 28 per cent of the total sulfur present

TABLE I  
*Oxidation of Cystine Sulfur to Sulfate Sulfur by Nitrous Acid*

Duration of reaction	Series 1			Series 2		
	Total S of cystine	Weight of BaSO <sub>4</sub> obtained	Sulfate S of total S taken	Total S of cystine	Weight of BaSO <sub>4</sub> obtained	Sulfate S of total S taken
hrs.	mg.	gm.	per cent	mg.	gm.	per cent
0.25	51.32	0.1046	28.0	51.32	0.1061	28.5
		0.1049			0.1067	
0.50	51.32	0.1253	33.6	48.88	0.1247	35.1
		0.1259			0.1252	
1.50	51.32	0.1380	36.9	48.88	0.1380	38.7
		0.1379			0.1377	
3.00	51.32	0.1468	39.3	48.88	0.1426	40.1
		0.1468			0.1430	
6.00	48.88	0.1598	45.0	48.88	0.1550	43.6
		0.1602			0.1550	

was oxidized. No extra barium nitrite was added in these experiments.

Since the preliminary experiments had indicated that the extent of oxidation of the sulfur of cystine could be increased somewhat by the addition of more barium nitrite to the reaction mixture after some hours, an attempt was made to determine the maximum oxidation to sulfate sulfur which could be obtained by repeated addition of fresh nitrite. Two such series of experiments extending over 84 and 144 hours respectively were carried out in duplicate. In the first series, seven pairs of beakers containing the cystine, acid, and nitrite were prepared and incubated at

25° as outlined. After 12 hours, the contents of one pair of beakers were filtered off and the barium sulfate weighed. To each of the remaining beakers, 1.62 gm. of crystalline barium nitrite were added. After an additional 12 hour period, the contents of two more beakers were filtered off and the sulfate weighed. The remaining beakers received the same amount of barium nitrite as before. This procedure was continued until with the last pair of beakers 84 hours had been available for the oxidation and six extra portions of the nitrite had been added at 12 hour intervals. The acidity was regulated by the addition of hydrochloric acid (1:1) as required to keep the reaction definitely acid to litmus. The procedure of the second series which extended over a total of 144 hours or twelve 12 hour periods was similar. Despite the repeated addition of a fresh supply of the reagent at intervals, the rate of oxidation slowed up rapidly in each series and slight oxidation occurred after 72 hours. Under these conditions it was not possible to oxidize the sulfur of cystine completely to sulfate sulfur, a maximal oxidation of about 85 per cent of the sulfur being obtained in 144 hours.

The experimental procedures already described were applied to a study of the oxidation of a number of sulfur-containing compounds related to cystine. In those experiments in which the period of oxidation was of 24 hours duration, 1.62 gm. of crystalline barium nitrite were added to each beaker at the end of 12 hours. At the same time, the behavior of these compounds in the Van Slyke gasometric method for amino nitrogen was determined. The results are presented in Table II.

In the Van Slyke method, cystine yielded 15 to 20 per cent of nitrogen in excess of the amount present in the amino groups. These values are somewhat higher than those obtained by Van Slyke (1), but are probably to be explained in part by the longer periods of reaction and the higher temperature at which the reaction was carried out in our studies. Ready oxidation of the sulfur of the cystine occurred. When the carboxyl groups of cystine were esterified (*e.g.* cystine dimethyl and diethyl ester hydrochlorides), the reaction of the amino group with nitrous acid appeared to be delayed slightly in the short reaction period, although in the longer periods, amounts of amino nitrogen considerably in excess of those present were indicated. The sulfur of these ester hydrochlorides

was readily oxidized to sulfate by nitrous acid and to approximately the same extent as was the sulfur of cystine. When cystine had been oxidized to a sulfonic acid derivative, cysteic acid

TABLE II

*Oxidation of Sulfur of Cystine and Related Sulfur Compounds by Nitrous Acid and Amount of Nitrogen Obtained from These Compounds by the Van Slyke Nitrous Acid Reaction for Amino Acids*

In the case of compounds which contained no nitrogen, or which contained nitrogenous groups not reacting as primary amines with nitrous acid (e.g., diacetylcystine), all the nitrogen obtained (Van Slyke) was "extra nitrogen." The percentages given were calculated by the expression, (gm.-atoms of N (Van Slyke)/gm.-atoms of S in sample)  $\times$  100.

Compound	Oxidation of S by nitrous acid Sulfate S of total S		N by Van Slyke procedure Amino N of total N		
	12 hrs.	24 hrs.	3 min.	30 min.	60 min.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Cystine.....	46.8*	57.5	114.9	117.7	120 0
"    dimethyl ester hydrochloride.....	42.4	47.3	91.0	111.0	
Cystine diethyl ester hydrochloride					
Sample 1.....	39.7	46.7	94.8	116.5	
"    2.....	40.5	48.4	93.2	114.3	132 4
Cysteic acid.....		0.5	100.1	99 0	
Taurine.....		0.0	97.4	95.6	
S-Benzylcysteine.....	2.1	5.3	98.3	100.0	102.4
Diacetylcystine.....	4.1	7.0	1.6	1.9	1.5
Barium $\beta,\beta'$ -dithiodiglycerate					
Synthetic, Sample 1†.....	6.4	10.7	0.0	0.0	0.0
"    "    2†.....	5.0	8.4	0.0	0.0	2.7
From cystine (5).....	7.3	11.4	5.1	6.1	5.8
dl-Methionine.....		0.0	100.2	101.0	100.3
Thioglycolic acid.....	89.0	94.6	1.1	11.3	41.5§

\* In 6 hours, 44.3 per cent of the sulfur was oxidized to sulfate.

† Prepared by Koelsch (7).

‡ Prepared in this laboratory by the method of Koelsch (7).

§ In a 2 hour period 55.0 per cent.

or taurine, neither formation of extra nitrogen nor oxidation of the sulfur by nitrous acid was to be observed (Table II). When the thiol group of cysteine was blocked by a benzyl group, as in S-

benzylcysteine, no extra nitrogen was obtained and the oxidation of the sulfur was so slight as to be almost negligible. Similar results were obtained with S-phenylcysteine. When methionine reacted with nitrous acid, no extra nitrogen and no oxidation to sulfate resulted. This was not unexpected in view of the findings with S-benzylcysteine. When the amino group was protected by an acetyl group (*e.g.* diacetylcystine), no significant amount of extra nitrogen and no oxidation of the sulfur to sulfate were observed.

The results obtained indicated a direct relation between extra nitrogen and oxidation of the sulfur to sulfate in the reaction with nitrous acid. A study of the behavior of compounds containing a thiol group but free from nitrogen afforded further evidence of this relationship. Thioglycolic acid (Table II) which contains no nitrogen gave more extra nitrogen than did any other compound studied and its sulfur was more completely oxidized, a conversion of 89 per cent to sulfate in 12 hours. Similar although slightly less striking results were obtained with  $\alpha$ -thiolpropionic acid by Miss Wilma Marlowe in unpublished results from this laboratory. She also noted that in the reaction with thiodiglycolic acid, a compound which does not contain the thiol group and which does not yield this group readily on reduction, neither nitrogen nor sulfate was formed.

Schmidt (2) has reported that  $\beta$ ,  $\beta'$ -dithiodiglyceric acid (desaminocystine) yielded extra nitrogen in the Van Slyke reaction, although in amounts much less than cystine itself. His compound, prepared from cystine by the action of nitrous acid, was not entirely pure. We have prepared the barium salt of this acid from cystine (3) and have studied its behavior, since the barium salt is more readily obtained in pure condition than the acid and since the presence of the barium was not objectionable in our procedure. The salt contained 35.95 per cent of barium (theory, 36.34 per cent) and 16.79 per cent of sulfur (theory, 16.99 per cent). When this compound reacted with nitrous acid, slightly less than 6 per cent of extra nitrogen was obtained. It will be noted, however, that contrary to the observations made with other sulfur compounds which reacted to give extra nitrogen, there was no significant increase in the amount of extra nitrogen as the reaction period was extended. Slight oxidation to sulfate was observed with the



formation of amounts of sulfate which we believe to be greater than the error of our experimental method. Due to the uncertainty as to the exact nature of the so called desaminocystine, we repeated our experiments with the barium salt of  $\beta, \beta'$ -dithiodiglyceric acid synthesized according to the method of Koelsch (7) using compounds prepared by Koelsch<sup>1</sup> and also in our own laboratory. Neither of these synthetic products yielded nitrogen in the reaction with nitrous acid. There was formed a small amount of sulfate, but the amount was clearly much less than the amount formed in the reaction between cystine or thioglycolic acid and nitrous acid. It is possible that the difference between the results obtained with the barium salt obtained from the deamination of cystine and the synthetic products may have been due to the presence of some substance other than the dithiodiglycerate in the deamination product. It seems possible that the  $\alpha$ -keto derivative of cystine might be formed, in part at least, rather than the  $\alpha$ -hydroxy derivative. Such a derivative, which differs from the  $\alpha$ -hydroxy compound only in containing 4 less hydrogen atoms, would give essentially the same analytical values as the hydroxy compound. Since the sulfur of cystine is readily oxidized to sulfate by the action of nitrous acid, the failure of the sulfur of the  $\beta, \beta'$ -dithiodiglyceric acid to undergo a similar ready oxidation to sulfate suggests that at 25° this compound may not be the chief product of the reaction of cystine with nitrous acid. It is of interest to recall in this connection, that glycolic acid, the hydroxy derivative formed by the deamination of glycine, did not react with nitrous acid to form nitrogen (1), although glycine yielded an amount of nitrogen in excess of that present in the amino group.

Since the results had supported the hypothesis that extra nitrogen is obtained from sulfur compounds related to cystine only when oxidation of the sulfur to sulfate occurs, it seemed desirable to determine whether the gas was actually nitrogen or some other substance. It is obvious that a compound containing an amino group (*e.g.* cystine), which, when acted upon by nitrous acid should yield nitrogen, could not be used to establish the identity

<sup>1</sup> We wish to express our indebtedness to Dr. Koelsch and Dr. MacElvain of the University of Wisconsin for placing this material at our disposal. Because of the small amount available, we were unable to analyze this sample.

of the gas. It has been shown (Table II) that in the presence of nitrous acid, thioglycolic acid, a nitrogen-free compound, yields a considerable quantity of a gas which is not absorbed by alkaline permanganate. Thioglycolic acid was therefore chosen for this study. If the gas produced proved to be nitrogen and if this nitrogen appeared to be formed simultaneously with the oxidation of the sulfur of thioglycolic acid to sulfate, it would seem probable that the nitrogen originated as a product of the reduction of the oxidizing agent, nitrous acid. Moreover, in view of the similarity of the sulfur linkages in thioglycolic acid and cysteine (readily formed from cystine) and of the fact that sulfur of both thioglycolic acid and cystine may be oxidized readily to sulfate by nitrous acid, it would seem logical to conclude that the behavior of cystine is similar to that of thioglycolic acid with respect to extra nitrogen formation.

The reaction of thioglycolic acid and nitrous acid was carried out with all precautions to exclude nitrogen from sources other than the reaction, gases other than nitrogen were absorbed by the alkaline permanganate in the Hempel pipette, the residual gas was transferred to a Geissler tube and examined spectroscopically.

The band spectrum of the gas was photographed at 10,000 volts alternating current in the region from 8000 to 3300 Ångström units with a Hilger quartz spectrograph. All the bands were attributed to the nitrogen molecule. In spite of the fact that the plate was subjected to long exposure, there was no evidence of the presence of bands to be attributed to carbon monoxide, nitric oxide, or sulfur dioxide. It would appear, therefore, that of the gases given off during the action of nitrous acid on thioglycolic acid, the only gas not absorbed by alkaline potassium permanganate is nitrogen. This observation lends strong support to the belief that the "extra nitrogen" obtained from cystine in the Van Slyke reaction is nitrogen in reality.

An attempt is being made to measure simultaneously the extra nitrogen and the sulfate sulfur formed from the reaction of cystine and nitrous acid. If this attempt is successful, it should then be possible to depict more accurately the quantitative aspects of the reaction which forms the subject of this paper.

The spectrographic examination of the gas was made by Dr. R. W. Smith of the Department of Physics, to whom we wish to express our indebtedness.

## SUMMARY

1. In an effort to explain the anomalous behavior of cystine in the Van Slyke gasometric method for amino nitrogen (1, 2), a study was made of the reaction of a series of sulfur-containing compounds with nitrous acid.

2. The sulfur of cystine was oxidized rapidly to sulfate by nitrous acid, but it was impossible to effect the oxidation of more than 85 per cent of the sulfur, even over a period of 144 hours and with the frequent addition of a fresh supply of the oxidizing agent.

3. Evidence was obtained to support the contention of Schmidt (2) that the extra nitrogen produced in the reaction between cystine and nitrous acid is related directly to the oxidation of the sulfur to sulfate with the reduction of nitrous acid to nitrogen.

4. When thioglycolic acid reacted with nitrous acid, the gas not absorbed by the alkaline permanganate in the Van Slyke determination was shown by spectroscopic examination to be nitrogen. Since thioglycolic acid resembles cystine in the Van Slyke reaction in that extra nitrogen is produced and the sulfur is rapidly oxidized to sulfate, it is believed that the "extra nitrogen" in the reaction between cystine and nitrous acid is in reality nitrogen, formed as a result of the reduction of the acid simultaneously with the oxidation of the sulfur.

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## THE EFFECT OF ALKALI ON THE TESTICULAR HORMONE\*

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The present authors have previously reported certain chemical properties of the testicular hormone obtained from extracts of bull testes and human urine (1-3). Most noteworthy of these findings was the rapid destruction of the hormone from bull testis tissue following treatment with boiling alkali. We were therefore surprised to find that the literature revealed an almost universal use of alkaline hydrolysis in some stage of the extraction or purification of the male hormone. Thus Dodds, Greenwood, Allan, and Gallimore (4, 5) employ  $\text{Ba}(\text{OH})_2$  hydrolysis in the preparation of a water-soluble form of the active principle from bull testes; Dingemans, Freud, Kober, Laqueur, and Münch (6) mention the use of alkaline hydrolysis with testicular extracts; Funk and Harrow (7) saponify their urinary extracts; Butenandt (8) makes the statement that the male hormone resists alkaline hydrolysis. The last worker probably limits his statement to urinary extracts since he has not reported extensively on testicular extracts.

The divergence of our results from those of other workers in the field led us to an exhaustive investigation of this phase of male hormone chemistry.

### EXPERIMENTAL

In these studies, testicular extracts were prepared and assayed by the authors' methods (3, 9). The preparation of our urinary extracts is described elsewhere (2). Our Preparations  $\Theta\text{OA}$  (0.10 mg. = 1 bird unit) and  $\Theta\text{SCE}$  (0.097 mg. = 1 bird unit) from bull testes, and Preparations  $\text{UIIHE}$  (0.24 mg. = 1 bird unit) and  $\text{XM}$  (0.52 mg. = 1 bird unit) from human urine were employed.

\* These studies were supported in part by a grant from the Committee for Research in Problems of Sex of the National Research Council and Biological Grant No. 1 from the Rockefeller Foundation.

*Preparation CT1*—5 cc. of stock solution (6.0 mg. = 600 bird units) were added directly to 10 cc. of freshly prepared 5 per cent alcoholic KOH and refluxed in a boiling water bath for 30 minutes; the alcohol was rapidly removed under diminished pressure, and the residual solution immediately acidified strongly with 10 per cent  $\text{H}_2\text{SO}_4$ . The material was transferred to a separatory funnel and extracted with ether four times. The acid aqueous layer was discarded because previous studies had shown this to be inactive. The combined ether extracts were washed four times with distilled water, then concentrated under diminished pressure, and the residue dissolved in alcohol to a volume of 25 cc.

*Preparation CT2*—5 cc. of stock solution (6.0 mg. = 600 bird units) were added directly to 10 cc. of freshly prepared 5 per cent alcoholic KOH and treated as above, but the treatment with KOH was continued for 1 hour. The final volume in alcohol was 25 cc.

*Preparation CT2a*—9 cc. of the 25 cc. of alcoholic solution of Preparation CT2 were evaporated to dryness. Next 5 cc. of 95 per cent ethanol and 10 cc. of 7.5 N HCl were added, refluxed for 2 hours, evaporated, and extracted as for Preparation CT1. The final volume in alcohol was 25 cc. If no hormone had been destroyed or lost in the preparation of Preparation CT2, this product would have started with 215 bird units. Actually the assay of Preparation CT2 showed that only 105 bird units remained after the alkali treatment. The purpose of this experiment was to determine whether acid treatment regenerated the lost activity.

*Preparation CT3*—5 cc. of stock solution (6 mg. = 600 bird units) were added directly to 10 cc. of freshly prepared 5 per cent alcoholic KOH and the alcohol immediately removed under diminished pressure at 40°. The residue was acidified and extracted as for Preparation CT1.

*Preparation CT3a*—15 cc. of Preparation CT3 (360 bird units if there was no destruction involved in Preparation CT3; by actual assay 385 bird units were found) were evaporated to dryness and 5 cc. of 95 per cent ethanol and 10 cc. of freshly prepared 5 per cent alcoholic KOH were added. After refluxing for 180 minutes in a boiling water bath, the alcohol was removed as usual. The residue was acidified, and extracted as for Preparation CT1. The final volume in alcohol was 25 cc.

*Preparation CT7*—5 cc. of stock solution (6.0 mg. = 800 bird units) were added to 10 cc. of aqueous 7.5 N HCl, refluxed for 120 minutes in a boiling water bath, and the alcohol removed under diminished pressure. The residue was acidified and extracted as for Preparation CT1. The final volume in alcohol was 25 cc.

*Preparation CT5*—2 cc. of Preparation XM (364 mg. = 700 bird units) were treated exactly as was Preparation CT2. The final volume in alcohol was 25 cc.

*Preparation CT6*—2 cc. of Preparation XM (364 mg. = 700 bird units) were treated exactly as Preparation CT3. The final volume in alcohol was 25 cc.

*Preparation CT14*—5 cc. of Preparation UIIHE (135 mg. = 550 bird units) and 7 cc. of Preparation  $\Theta$ SCE (47.6 mg. = 490 bird units) were mixed together, 8 cc. of freshly prepared 10 per cent alcoholic KOH added, and the mixture refluxed on a steam bath at boiling temperature for 240 minutes. The alcohol was quickly removed, the mixture acidified, and extracted as for Preparation CT1. The final volume in alcohol was 25 cc. The purpose of this experiment was to show that the urinary product does not contain an impurity which protects the male hormone against the action of the base.

*Preparation CT15*—2 cc. of Preparation UIIHE (54 mg. = 220 bird units) and 2 cc. of Preparation  $\Theta$ SCE (13.6 mg. = 140 bird units) were mixed together, 5 cc. of freshly prepared 10 per cent alcoholic KOH added, and the alcohol was rapidly removed without warming. The mixture was acidified and extracted with ether as for Preparation CT1. The final volume in alcohol was 25 cc.

*Preparation GK12*—In order to eliminate the effect of the hypothetical formation by alkali action of an inhibiting or antagonistic substance toward the male hormone as an explanation for the loss of activity after alkali action, a known number of bird units, not treated by base, were mixed with the same number which were first destroyed up to 91 per cent by base, and the mixture was then assayed. The results on the mixture agreed with the calculated value from the separate assays. No antagonistic action was observed.

The alkali and acid treatments and assays were carried out by one of us (Gallagher). The flasks containing the treated samples

were given to Koch who prepared the dilutions for assay by adding the desired quantity of the alcoholic solutions to olive oil and heating under diminished pressure to remove the alcohol. These samples were given to the senior author as complete unknowns under separate numbers. Separate records were kept and at the termination of the assay comparison was made and the results calculated. In this way the study was made as objective as possible.

#### DISCUSSION

The results given in Table I are self-explanatory. The differences recorded are significant statistically as calculated from the standard error for each determination according to the table of Fisher (10). That the activity of the extracts from bull testes is destroyed by boiling with alkali cannot be disputed. In 1 hour at least half of the activity is lost and at the end of 3 hours approximately 90 per cent has disappeared. The control adequately proves that the activity is not lost through handling. The experiment with Preparation CT2a proves that the activity cannot be regenerated from the alkali-treated material by subsequent treatment with acid. The assay of Preparation GK12 proves that the loss of activity does not involve the production during alkali treatment of substances antagonistic to the male hormone action, since in the presence of a large amount of alkali-treated extract added quantities of untreated hormone are quantitatively detected. At the same time it is quite evident that the activity of urinary preparations is not diminished by similar treatment. Here indeed is an astonishing chemical difference between the hormone of the testis and the active principle found in urine. That these differences in resistance are inherent in the compounds themselves and are not due to protecting impurities in urine is shown by experiments on Preparations CT14 and CT15. Bull testis extracts treated with alkali in the presence of urinary extracts lose potency at about the same ratio as when treated alone. The control shows that the added testis hormone is quantitatively detected in the presence of urinary extract. This experiment is really the crucial test of a difference between the hormones from the two sources.

Our own work on the biological activity of urine preparations by

comb growth tests as well as the response of the mammalian secondary sex characters indicates that the urinary material possesses all the biological activity that is found with testicular extracts. The results of other workers in the field likewise indicate that the urinary and testicular extracts have the same biological activity. The biological identity is further strengthened

TABLE I  
*Showing Bird Units Taken, Treatment, and Bird Units Recovered*

Preparation	Taken from		Treatment	Time	No. of birds used for assay	Found by assay after treatment	Loss
	Testis tissue	Human urine					
	<i>bird units</i>	<i>bird units</i>		<i>min.</i>		<i>bird units</i>	<i>per cent</i>
CT1	645	0	Boiled in 3.3% alcoholic KOH	30	18	235	63
CT2	645	0	Same	60	23	290	55
CT2a	105	0	CT2 boiled in 5 N HCl	120	17	90	15*
CT3	645	0	Control test	0	24	645	0
CT3a	385	0	CT3 boiled in 3.3% alcoholic KOH	180	8	35	90
CT7	645	0	Boiled in 5 N HCl	120	18	570	10*
GK12	55	0	CT3a (5 b.u.)		4	60	0
			CT7 (50 b.u.)				
CT5	0	705	Boiled in 3.3% alcoholic KOH	60	17	815	15* gain
CT6	0	705	Control test	0	27	705	0
CT14	490	550	Boiled in 4.0% alcoholic KOH	240	15	680	35 (73% of testis tissue hormone)
CT15	140	220	Control test	0	6	350	4*

\* These are not significant values.

by various other researches. These results leave little room for doubt that the two substances possess in some degree at least the same biological action (2).

This research clearly indicates that the active principles extracted from bull testes and from human male urine are chemically two distinct substances. Whether the substance extracted from



the testis is a precursor of the hormone circulating in the blood or whether the hormone is altered to the form found in the urine by its passage through the animal body is, of course, a debatable problem. That there is a species difference in the hormone obtained from cattle and that from the human male would not be impossible but by analogy seems unlikely. This is under investigation now. The assumed or postulated conversion of the more labile form to the more stable urinary product with retention of physiological activity is very unusual.

The chemical change involved in the destructive action of alkali on the hormone of the testis tissue is pure speculation at the present time. It certainly appears to be a non-reversible change involving two or more new products or a simple molecular rearrangement. Whether oxidation is involved has not been determined. There is no evidence at present to lead to the interpretation that the destruction involves the saponification of a hormone ester or that it involves a typical racemization of an active form to a less potent tautomer. The questions raised here will be most satisfactorily answered through the isolation of the testis hormone from testis tissue.

#### SUMMARY

1. The male hormone extracted from bull testis tissue is destroyed by boiling with alkali.
2. The male hormone extracted from human male urine is not destroyed by the same treatment.
3. Control experiments prove that the instability of the one and the stability of the other are not due to the presence of impurities in the two preparations.
4. The nature of the chemical change in the destructive action by alkali is not known.

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## THE SPECIFIC EFFECTS OF BUFFERS UPON UREASE ACTIVITY\*

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(Received for publication, January 17, 1934)

Since nearly all of the previous measurements of urease activity have been carried out in the presence of phosphate buffer, it has not been generally recognized that the activity of urease is greatly influenced by the type of buffer employed; indeed the results of earlier workers are a measure of the effect of phosphate upon urease as well as a measure of the effect of urease upon urea. Krebs and Henseleit (1) state that at pH 5 phosphate buffer inhibits urease action and Folin (2) has recently preferred to employ acetate buffer in the analysis of urea by urease in blood and urine. It may be recalled that phosphate has been found to inhibit saccharase (3-5), catalase (6), peroxidase (6), and amylases (7, 8).

We have investigated the effect of acetate, citrate, and phosphate upon crystalline urease from the jack bean. With these three buffers we have obtained urea concentration curves for urease at several pH values. As is shown in Fig. 1 the activity of urease in phosphate buffer at a given pH increases until an optimum urea concentration is reached, after which the activity decreases. This effect depends upon the pH of the buffer, for when the pH is below pH 6.0 there is no inhibition of urease activity when as much as 10 per cent urea is used, while if the pH of the buffer is above pH 6.0 the amount of urea required to inhibit urease activity decreases with decreasing acidity. The

\* This is taken from the report of one of us (S. F. H.) submitted to the faculty of the Graduate School in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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curves also show that the urea concentration for optimum activity increases with increasing acidity. At pH 5.0 the optimum urea concentration with phosphate buffer is calculated to be about 20 per cent; at 5.6 it is 10 per cent; at 6.4 it is 5 per cent; at 6.7 it is 2.5 per cent; at 7.2 it is 1.5 per cent; and at 7.9 it is about 0.7 per cent.

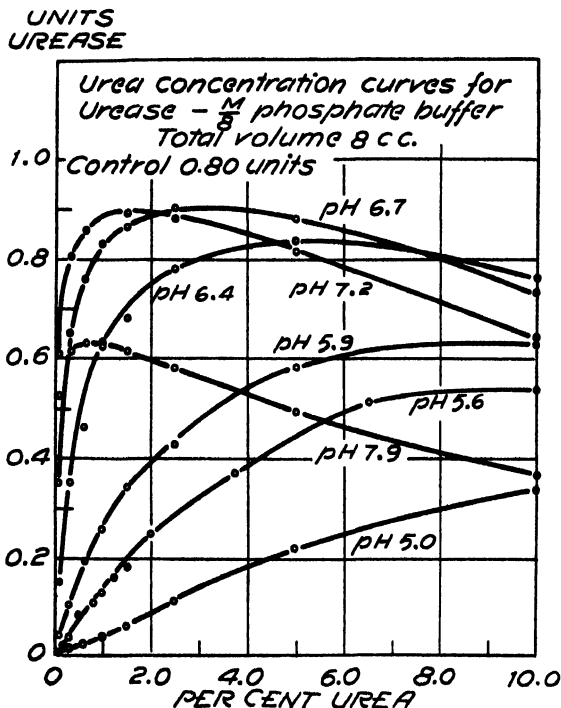


FIG. 1

The same general relationship as that described above holds for acetate and citrate buffers (Figs. 2 and 3), although with these two buffers the concentration of urea required for optimum activity is consistently lower. While with phosphate buffer optimum urease activity at optimum pH is with 2 per cent urea, in acetate and citrate buffers it is attained with 1 per cent urea.

In order to rule out the effect upon our results of buffer, or

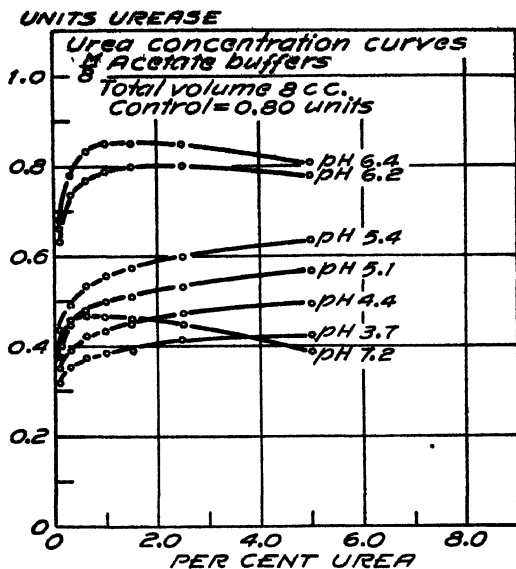


FIG. 2

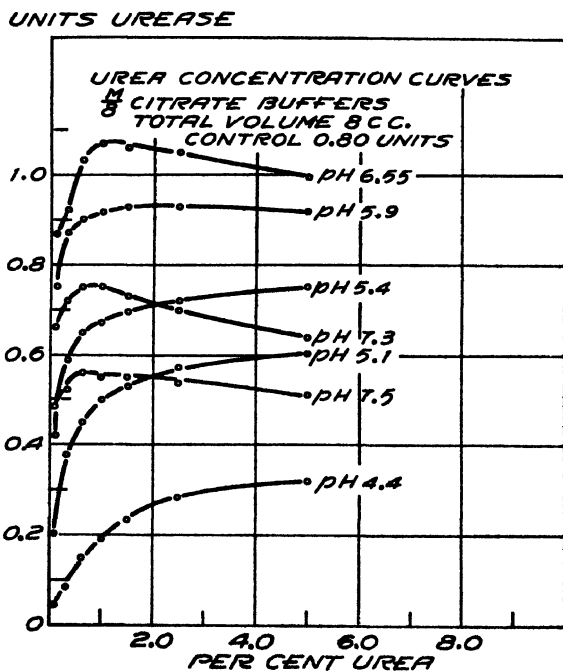


FIG. 3

salt concentration, we have obtained buffer dilution curves, which are shown in Fig. 4. Here two different urea concentrations were employed with the three buffers. The curves show that urease activity increases with increasing buffer dilution until a point is reached beyond which further dilution may cause the activity to decrease. With 0.1 per cent urea concentration the optimum dilution for all three buffers is  $m/8$ , but with 2.5 per cent urea it is

UNITS  
UREASE

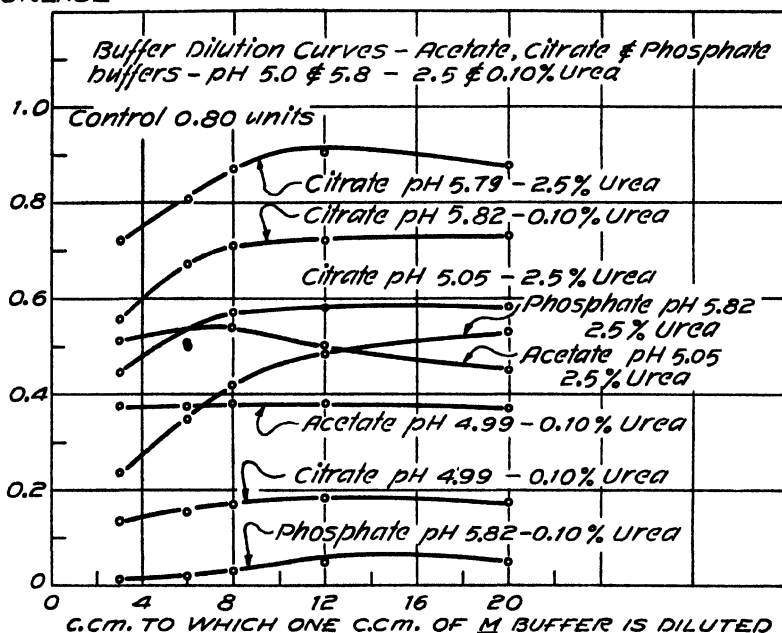


Fig. 4

$m/12$ . Van Slyke and Zacharias (9) reported an optimum phosphate buffer dilution at  $m/16$  at pH 7.0 with 0.91 per cent urea.

The specific effects of acetate, citrate, and phosphate buffers have been determined at various pH values, 0.1 and 2.5 per cent urea concentrations being used, as shown in Figs. 5 and 6. Here an entirely different activity-pH curve is obtained for each buffer. With phosphate urease is active from pH 5 to 9, with

citrate from pH 4 to 8.5, and with acetate from below pH 3 to 7.5. The optimum pH differs with each buffer as well as with urea concentration. With 2.5 per cent urea the optimum is at pH 6.4 for acetate, at 6.5 for citrate, and at 6.9 for phosphate; while with 0.1 per cent urea the values are shifted respectively to pH 6.7, 6.7, and 7.6. It is of interest to note that Ringer and van Trigt (10) found the pH optimum for ptyalin to be situated more towards

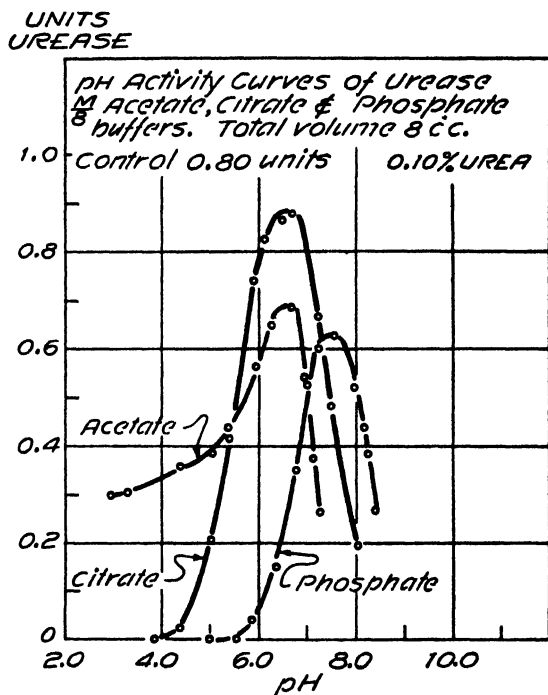


FIG. 5

the alkaline side in citrate buffer than in phosphate or acetate buffers. Hahn and his coworkers (7, 8) found the optimum for salivary amylase to be pH 5.6 in acetate buffer and 6.5 in phosphate. For pancreatic amylase the values were respectively 6.5 and 7.1. The pancreatic amylase was 3.3 times more active in phosphate than in acetate. The greatest activity of urease has been attained with citrate buffer at pH 6.5 and with a urea con-



centration of 1 per cent. In this connection it is of interest to note that the pH optima for urease at various urea concentrations have been repeatedly misstated (11-14).

It has already been noted by Van Slyke and Zacharias (9) and by Lövgren (15) that the pH optimum for urease shifts toward the alkaline side with decreasing urea concentration. Although this shift is considerable with phosphate buffer, it is slight with both acetate and citrate.

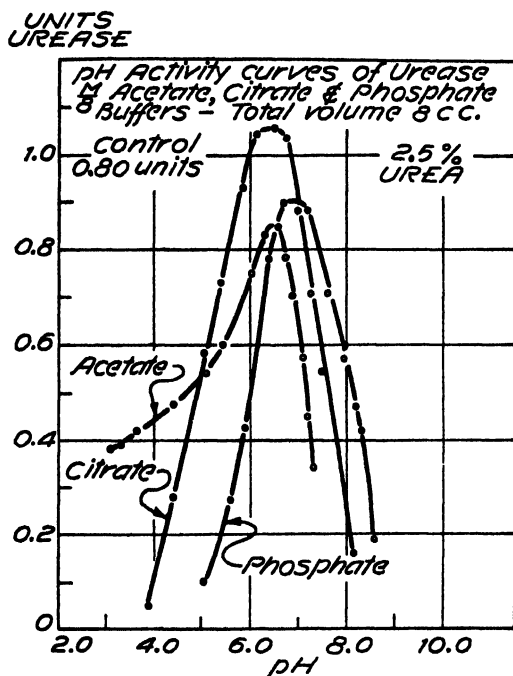


Fig. 6

The activity-pH curve for urease in acetate buffer is quite different in the extreme acid range from that of the other two buffers, for the curve with acetate shows no sharp drop with increasing acidity.

#### EXPERIMENTAL

For each measurement of urease activity three analyses have been run, an activity determination, a control, and a blank. In

addition the pH has been determined each time both before and after incubation with urea. Urease activity was determined by adding 1 cc. of diluted urease to 7 cc. of urea-buffer contained in a large test-tube and kept at 20° in a thermostat bath. The digestion was allowed to proceed for 1 minute and was then stopped by the addition of 3 cc. of N hydrochloric acid. The ammonia formed was determined by aeration and titration. When the determination of pH was to be made urease action was stopped by adding quinhydrone. The pH values in all cases are averages of the pH before and after urease action.

The urease used was recrystallized once from 30 per cent alcohol and stock solutions were made up containing 1200 units per cc. For daily use 1 cc. was diluted 400 times. The urea and acid potassium phosphate were especially purified by us, while other reagents were Kahlbaum's. All water used was redistilled from glass.

#### SUMMARY

1. The activity of urease depends upon the type of buffer present as well as upon temperature, pH, urea concentration, and salt concentration.

2. The pH optimum for urease acting upon 2.5 per cent urea is 6.4 for acetate, 6.5 for citrate, and 6.9 for phosphate. With 0.1 per cent urea the optimum is 6.7 for acetate, 6.7 for citrate, and 7.6 for phosphate.

3. The highest activity is exerted by urease in the presence of 1.0 per cent urea and M/8 citrate buffer at pH 6.5.

4. In phosphate buffer urease is active from pH 5 to 9, in citrate buffer from pH 4 to 8.5, and in acetate buffer from below pH 3 to 7.5.

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## **AN IMPROVED METHOD FOR THE DETERMINATION OF ACETYL VALUES OF LIPIDS APPLICABLE TO HYDROXYLATED FATTY ACIDS\***

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None of the methods that have been proposed for the determination of the acetyl values of lipids is generally satisfactory and none is applicable to free hydroxylated fatty acids. Benedict and Ulzer (1) first proposed a method in which the insoluble fatty acids of a fat are acetylated and the acetyl value determined by estimating the KOH bound before and after saponification of the acetylated acids. Lewkowitsch (2), however, showed this method to be inaccurate and to give high results by demonstrating that non-hydroxylated acids, as palmitic, stearic, and oleic, give considerable acetyl values when treated according to Benedict and Ulzer's method. This he found to be due to relatively stable mixed anhydrides formed by boiling the acids with acetic anhydride. Lewkowitsch (3) then proposed the method which in essentials is the one most commonly used at the present time, and which consists in acetylating the fat (or other lipid, not fatty acids) by boiling with acetic anhydride, removing excess anhydride by boiling with water, and determining the acetic acid liberated (as mg. of KOH equivalent) per gm. of acetylated material. A determination of volatile acids must be run on the original unacetylated material and subtracted from the above results to give the so called true acetyl value. The method is laborious and time-consuming as well as subject to a number of errors. If the material acetylated contains free fatty acids, the results may be too high, as in the

\* Presented in part before the meeting of the American Society of Biological Chemists at Cincinnati, April, 1933.

method of Benedict and Ulzer. The prolonged boiling of the acetylated product with water may lead to hydrolysis and loss of acetic acid with low results (4). The titrations of small amounts of acid in the large volumes required are not accurate. This is an important source of error when the acetyl value is low and the volatile acid correction of rancid oils may be equal to or greater than the true acetyl value.

Roberts and Schuette (4) have recently published an acetyl method which consists in heating the sample with a weighed amount of standardized acetic anhydride in a sealed tube at 120° for an hour, followed by refluxing at reduced acidity, and final titration with alkali. The writers have found the method tedious and have been unable to obtain consistent results with it.

The evident need, stressed by Andrews and Reed (5), for a simple accurate method applicable not only to fats, waxes, and sterols but also to *free hydroxylated fatty acids* has prompted the writers to devise the procedure reported below. It is being used in this laboratory in a study of the distribution of hydroxylated fatty acids in the animal body and their metabolism.

Verley and Bölsing (6) determined the hydroxyl groups of alcohols and phenols by acetylating with acetic anhydride and pyridine, decomposing excess anhydride with water, and titrating with aqueous alkali. Peterson and West (7) showed the method to be applicable to hydroxyl groups of sugars and sugar derivatives. A thorough study of the method in attempts to apply it to accurate determination of the acetyl values of lipids in this laboratory has failed, except with castor oil and some of the waxes. The failure was found to be due to the presence of free fatty acids in the acetylated mixtures which could not be efficiently removed by filtration and which do not titrate accurately in aqueous solution, especially in the large volumes necessary. These difficulties have now been overcome in the procedure outlined below.

The method consists in acetylating the sample with a measured quantity of a mixture of acetic anhydride and pyridine (either hot or cold), decomposing excess anhydride with a little hot water, and titrating with alcoholic alkali after the addition of sufficient butyl alcohol to give a homogeneous solution. The acidity of the material is determined in the same way on a sample treated with pyridine only and this value is subtracted from the above titration.

This result subtracted from the titration of a blank on the acetic anhydride-pyridine mixture gives the necessary data for calculating the acetyl value. The titration of all of the acids, both acetic and higher fatty acids, is very accurate when carried out in the butyl alcohol solution with alcoholic alkali. A large number of determinations may be run simultaneously and the execution of the method leaves little to be desired from the standpoint of simplicity, accuracy, and time required. Preliminary experiments indicate that the method may be applied satisfactorily to semi-microquantities.

The definition of acetyl value as "the milligrams of KOH required to neutralize the acetic acid from 1 gm. of acetylated fat"

TABLE I  
*Acetyl Values of Lipids*

Substance	Acetyl value by the Lewkowitsch definition	Acetyl value by proposed definition*	Substance	Acetyl value by the Lewkowitsch definition	Acetyl value by proposed definition*
Castor oil. ....	146.1	126	Olive oil.....	3.5	2.7
Cottonseed oil . . .	5.5	4.3	Carnauba wax....	41.2	32.6
Linseed oil.....	6.3	5.0	Cholesterol.....	130.9	111.5
Croton oil.....	8.3	6.4	Ricinoleic acid. .	160.1	134.7
Neat's-foot oil....	20.0	15.4	Lithium lactate ..	395.3	431.7
Coconut oil.....	4.4	3.4			

\* According to the proposed definition, acetyl value = mg. of acetyl ( $\text{CH}_3\text{CO}$ ) taken up per gm. of lipid = (titration difference (cc. of 0.1 N alkali)  $\times$  4.3)/(weight of sample in gm.).

is not satisfactory as a general definition. It has specific reference to the Lewkowitsch procedure of determining the acetyl value, and the necessity of calculating the weight of acetylated material when other methods are used is an unnecessary inconvenience. Calculation of the number of hydroxyl groups in a compound of known molecular weight from such acetyl values is also somewhat complicated. The writers propose as a more suitable definition of acetyl value, *the mg. of acetyl taken up per gm. of substance*. Such values have a definite chemical meaning which should be applicable to all methods of determination and easily used in related calculations. A comparison of values according to the present and proposed definitions is given in Table I.

*Methods**Reagents*

**Pyridine.** Mallinckrodt's medicinal grade is dried by refluxing for several hours with good barium oxide and then distilled. The fraction passing over above  $114^{\circ}$  is used.

**Acetic anhydride,** Mallinckrodt's reagent quality, redistilled.

**Alcoholic NaOH.** This is prepared by dissolving sufficient 60 per cent aqueous NaOH (60 gm. per 100 cc.) in 95 per cent alcohol to make a 0.3 to 0.35 N solution. The precipitated carbonate is easily removed by adding a little norit, shaking, and filtering. It should be standardized against standard acid (phenolphthalein) daily. The solution remains colorless for a long time if kept below  $25^{\circ}$ .

**Butyl alcohol,** ordinary commercial butyl alcohol (Commercial Solvents Corporation). It generally contains a trace of acid but this is taken care of in the blank titration.

*Procedure*

Two samples (0.5 to 1.0 gm. of materials with high acetyl values, as castor oil, and 2 to 3 gm. of those having low acetyl values, see Table II) of material are weighed into each of two 250 cc. ground glass-stoppered Pyrex Erlenmeyer flasks.

5 cc. of acetic anhydride-pyridine mixture (1 volume of anhydride and 7 volumes of pyridine) are added to one of the above samples from a Folin-Ostwald blood pipette, with careful measurement. To the other, 5 cc. of pyridine only are added. A blank flask is set up with 5 cc. of the acetic anhydride-pyridine mixture. The stoppers are moistened with pyridine and placed loosely in the flasks. The flasks are then placed over well fitting holes (to insure a minimum escape of steam around the flasks we have used rubber rings over the holes) of a steam bath, and allowed to heat about 5 minutes to permit expansion, the stoppers being slightly unseated if necessary. The stoppers are then turned in firmly and the heating continued 40 to 45 minutes.

The flasks are removed from the bath and the stoppers quickly loosened (to prevent sticking due to cooling) and placed at an angle in the mouths of the flasks. 5 cc. of water are then added, care being taken to rinse well the stoppers and necks of the flasks. The stoppers are loosely replaced and the flasks heated 1.5 to

TABLE II

*Acetyl Values of Lipids, Etc., by Pyridine-Acetic Anhydride Titration Method*

Substance	Temperature	Weight of sample	Acetyl values (in order of sample)	Average	Given in literature
		gm.			
Castor oil.....	Hot	0.596, 1.155	146.1, 144.6	145.3	146 -150.5
“ “ .....	Cold	0.630, 1.226	147.1, 147.3	147.2	
Cottonseed oil, fresh.....	Hot	2.069, 1.542, 2.940	5.3, 5.5, 5.4	5.4	5.5- 25
“ “ .....	Cold	1.383, 1.835	5.9, 5.2	5.5	
Cottonseed oil, old.....	“	3.670, 2.083, 1.465	31.4, 30.6, 29.8	30.6	
Linseed oil, fresh. “	“	3.424, 2.515, 1.694	5.8, 6.2, 6.1	6.0	4 - 8.5
“ “ “ .....	Hot	2.692, 1.770, 3.288	6.3, 6.5, 6.3	6.36	
Croton “ .....	Cold	3.156, 2.264, 1.563	8.2, 8.8, 8.7	8.56	19.8- 32
“ “ .....	Hot	2.350, 3.044, 1.609	8.2, 8.3, 8.3	8.26	
Neat's-foot oil... “	“	3.055, 2.303, 1.545	20.4, 20.0, 19.8	20.1	22
Cocoanut oil, fresh.....	Cold	2.052, 1.974, 1.932	4.4, 4.1, 4.8	4.4	0.9- 12
Cocoanut oil, old. “	“	3.612, 2.482, 1.150	19.4, 20.4, 20.1	20.0	
Olive oil..... “	“	3.498, 2.556, 1.774	3.5, 3.9, 3.3	3.6	4.9- 10.6
Carnauba wax... “	“	2.822, 1.228	41.2, 40.9	41	44.7- 55.2
Cholesterol.....	Hot	0.3352, 0.3826	130.9, 133.5	132.2	131.1 (theory)
Ricinoleic acid... “	“	0.3401, 0.3339	158.5, 158.8	158.6	164.6 “
“ “ “ .....	Cold	0.3463, 0.3463	159.6, 160.1	159.8	
Lithium lactate .	Hot*	0.3734, 0.3000	394.4, 395.3	394.8	406.1 “
Palmitic acid.... “	“	0.4755, 0.3135, 0.3547	No acetyl value		None

\* Heated 65 minutes on the steam bath.



2 minutes on the steam bath, after which they are allowed to cool 10 minutes with the stoppers at an angle. The stoppers and necks of the flasks are rinsed down with 25 cc. of butyl alcohol (more if necessary to give a homogeneous solution) and the solutions titrated with 0.3 to 0.35 *N* alcoholic NaOH from a good 50 cc. burette, with phenolphthalein as indicator (3 to 4 drops of 0.1 per cent in alcohol). The titrations are all reduced to cc. of 0.1 *N* alkali.

Instead of heating on the steam bath, the stoppers of the flasks may be sealed in with pyridine and the flasks permitted to stand 24 hours or longer at room temperature and the procedure continued as outlined above. Table II shows that results obtained with heating and in the cold agree well. The values here are expressed according to the Lewkowitsch definition.

### *Calculations*

According to Lewkowitsch's definition, the acetyl value equals the mg. of KOH required to neutralize the acetic acid from 1 gm. of acetylated substance. If we let *A* represent the weight of the sample acetylated, *B* the acidity (cc. of 0.1 *N*) of the blank, *C* the acidity (calculated from titration of the sample treated with pyridine alone) of the sample used, and *D* the acidity of the acetylated sample, the calculations are as follows: acidity equivalent of anhydride bound,  $E = B - (D - C)$ ; weight of acetylated sample,  $F = A + (E \times 0.0042)$ ; mg. of KOH equivalent to acetyl bound,  $G = 5.61 \times E$ ; acetyl value =  $G/F$ .

According to the proposed definition, the acetyl value equals the mg. of acetyl taken up per gm. of substance. With the necessary symbols above the calculation is acetyl value =  $(E \times 4.3)/A$ .

### DISCUSSION

Our statement that the method is applicable to free hydroxylated fatty acids and not subject to the error of the Benedict-Ulzer procedure is based upon several points of evidence. We prepared ricinoleic acid of neutral equivalent 298.5 (theory 298.36) according to the directions of Rider (8) and determined its acetyl value by our method both with heating and at room temperature with good agreement between the two determinations as shown in Table II. Our values, however, are a little lower than the theoretical, amounting to about 97 per cent of the theory. This is

probably due to the presence of a small amount of impurities and possibly to a slight polymerization of the ricinoleic acid similar to that pointed out by Meyer (9). The fact that the results obtained with heating and in the cold checked within the limits of error of the method indicates that no undecomposable mixed anhydrides were formed, because if this were true the heated samples would have undoubtedly been most affected and given higher acetyl values. This source of error also would have caused the acetyl values to be higher than the theory instead of lower, as found. In order to check this point several samples of palmitic acid were run with heating and in every case no acetyl value was found, the titration values checking closely with the acidity of the anhydride plus that of the acid. We have found very low acetyl values on a sample of ricinoleic acid kept under conditions favoring polymerization.

Carnauba wax and lithium lactate did not dissolve well in the acetylating mixture, the latter rather gelatinizing. Despite this fact both were completely acetylated as shown by the concordant results on different sized samples. The value found for lithium lactate is 97.33 per cent of the theory. Lactic acid determination on the salt by oxidation to aldehyde (10) showed it to be 96.9 per cent pure. This result also shows the method to be applicable to hydroxylated acids.

In most of our determinations we have purposely used samples of widely varying weights. This was done in order that we might be sure that acetylation proceeded to completion. We consider this, along with the general agreement with published values recorded in the literature as sufficient check on the method. The acetyl value for castor oil is essentially a constant owing to the presence of a rather definite large proportion of hydroxylated acids. Lewkowitsch (11) has pointed out that this is not true for most fats or oils since they may contain sterols and mono- and diglycerides owing to rancidity in varying proportions in addition to hydroxylated fatty acids. Table II shows the large effect of rancidity upon the acetyl values of cottonseed and coconut oils.

We believe that the above method of analysis now makes it possible, by determination of the acetyl values of the fatty acids in fats, to arrive at values which may be considered as true characteristics. This is being investigated.

## SUMMARY

A new method for the determination of the acetyl values of lipids has been developed. It is simple, accurate, and rapid. The method differs from those previously proposed in that it is applicable to free hydroxylated fatty acids.

A new definition of acetyl value more suitable for calculation than the present one is proposed.

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# **A SIMPLE ADAPTATION OF KOLTHOFF'S COLORIMETRIC METHOD FOR THE DETERMINATION OF MAGNESIUM IN BIOLOGICAL FLUIDS\***

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## *Determination of Magnesium in Plasma*

Although Peters and Van Slyke (1) state that, "Thus far no clinical significance has been attached to changes in magnesium metabolism," the fact that Meltzer and Auer (2) demonstrated that raising the blood magnesium gives rise to coma, and that Kruse, Orent, and McCollum (3) have produced a form of tetany by diets poor in magnesium indicates the possibility that syndromes of hyper- and of hypomagnesemia may play a greater rôle in clinical medicine than has been suspected heretofore. The methods for the determination of blood magnesium (McCrudden (4), Marriott and Howland (5), Denis (6), Kramer and Tisdall (7), Hammett and Adams (8), Briggs (9), Yoshimatsu (10), Eichberg and Berg (11), and Greenberg and Mackey (12)) all require the precipitation of the magnesium as ammonium magnesium phosphate or as magnesium oxyquinoline, and a subsequent determination of either the ammonium magnesium phosphate itself, its phosphate component, or

\* The method and some of the results of the determination of magnesium and calcium in the blood represent a part of a thesis of E. R. Serles presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Minnesota. This investigation was completed with the support of funds granted by the Committee on Therapeutic Research, Council of Pharmacy and Chemistry, American Medical Association.

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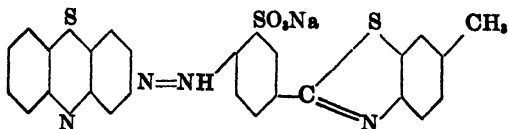
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of the excess of oxyquinoline. These determinations involve several steps and are time-consuming and rather cumbersome.

The method which we are reporting consists of a direct colorimetric determination of the magnesium in plasma or serum after the calcium has been precipitated out with oxalate, in other words in the supernatant fluid ordinarily thrown away after a Kramer-Tisdall calcium determination. Our method is based upon the discovery of Kolthoff (13) that magnesium imparts a pink or red color to alkaline solutions of two acridine sulpho dyes (titan yellow and Clayton yellow)<sup>1</sup> and that either of these dyes can be used for the colorimetric determination of small quantities of magnesium. As he found that calcium salts intensify this color, we have determined the magnesium in oxalated plasma; and as magnesium in amounts above 2 mg. per 100 cc. gives rise to lake precipitates with the dyes, we have prevented the formation of such lakes by adding a dispersing colloid (soluble starch or dextrin) to the standard magnesium solutions. This method can be used for microcolorimetry with 0.1 cc. of plasma.

After we had developed our method and had been using it for over a year for the determination of plasma magnesium, there appeared an article by Becka (14), who also attempted to adapt the method of Kolthoff for biochemical purposes. Becka used titan yellow, dispersed the magnesium-dye lakes with colloids (dialyzed agar, dextrin, etc.), and also used a yellow color screen as we have done. However, he adds the dye directly to plasma without precipitating the calcium, though Kolthoff had shown that the presence of calcium alters both the intensity and the nature of the color produced by magnesium. Becka thus introduces a considerable additional error, which, in known mixtures, we find to be 6.5 to 24.5 per cent, though these determinations are still further

<sup>1</sup> Titan yellow is manufactured by British Drug Houses, Ltd., London; Clayton yellow is manufactured by the National Aniline Company, Buffalo. Both these dyes are of the acridine sulpho group (acridine yellow SG) and have (approximately) the formula



vitiated because the colors do not match. In plasma, when calcium is not precipitated, the error is greater and may run as high as 82 per cent. This error is eliminated in our method.

### *Reagents*

The distilled water and the other reagents used for the test or for making up the reagents must be free from magnesium and calcium; *i.e.*, they must give no trace of pink when tested with NaOH solution plus a drop of the dye (titan yellow or Clayton yellow), and no precipitate or turbidity on the addition of ammonium oxalate. The ordinary laboratory distilled water from a single distillation in a block tin still has been satisfactory.

1. NaOH solution approximately 0.4 N (1.6 per cent) concentration. The NaOH itself must be free from magnesium and calcium. All the samples of a good grade of NaOH thus far tested have been satisfactory.

2. Dye solution. Titan yellow or Clayton yellow, 0.01 gm. per 100 cc. of  $H_2O$ . Either titan yellow or Clayton yellow may be used, but in any individual determination the same dye must be used with both plasma and standard, as the two dyes do not give perfect color matches with one another.

3. Ammonium oxalate, 3 gm. per 100 cc.

4. Colloid-dispersing agent. An approximately 0.5 per cent suspension of ordinary soluble starch or C.P. dextrin may be used, but the test described above for distilled water must show that the colloid is free from magnesium and calcium. The contents of a No. 1 gelatin capsule just filled with the powdered starch ( $411 \pm 7$  mg.) or dextrin ( $415 \pm 10$  mg.) may be taken as a sufficiently accurate amount, thereby saving the time of weighing. This amount is transferred to a mortar, ground up to a paste with a little cold  $H_2O$ , and then gradually diluted to 80 cc. The solution must be freshly prepared.

Other colloids have not proved satisfactory. Acacia, tragacanth, gum ghatti, and ordinary agar contain magnesium. Some specimens of electrodialyzed agar are free from magnesium but they impart a disturbing turbidity to the mixtures.

*Standard Magnesium Solutions*—For the determination of magnesium the color produced by diluted alkalized plasma or urine is compared with that produced by 0.0002 per cent magnesium (0.002 mg. of Mg per cc.). Such a standard magnesium solution used

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for the determination (Standard 2) is too dilute to be preserved and is therefore prepared freshly as described below.

5. Standard 1. A permanent standard solution containing 0.1 per cent Mg is prepared by weighing out exactly 1 gm. of powdered magnesium (C.P.), dissolving this in 5 cc. of concentrated HCl, and diluting to 1000 cc. with  $H_2O$ . This solution may be preserved for several months by adding 2 cc. of chloroform. Whenever the odor of chloroform has disappeared, 1 cc. of chloroform should be added and the bottle shaken vigorously (1 cc. = 1.0 mg. of Mg).

6. Standard 2. 2.0 cc. of Standard 1 are diluted to 100 cc. 1 cc. of this diluted standard is taken, 7 cc. of colloid-dispersing agent is added, then 1.0 cc. of 0.01 per cent titan (or Clayton) yellow solution, then 1.0 cc. of 0.4 N NaOH, the tube stoppered, and mixed thoroughly by inversion. This standard must be freshly prepared (1 cc. of the mixture = 0.002 mg. of Mg).

### *Analytical Procedure*

About 5 cc. of blood are placed in a 15 cc. centrifuge tube containing a few small crystals of sodium citrate. The tube is stoppered with a rubber stopper, the contents mixed thoroughly by inverting three times, and centrifuged at 1500 to 1800 R.P.M. for 15 minutes. 1 cc. of the plasma is pipetted off into another (preferably graduated) centrifuge tube, 0.5 cc. of 3 per cent ammonium oxalate solution added, and then 8.5 cc. of  $H_2O$  at 45°. Water at this temperature accelerates the crystallization and precipitation of the calcium oxalate without precipitating the globulins. The tube is stoppered, the contents mixed thoroughly by inversion, the tube kept in a water bath at 45° for 20 minutes, then centrifuged for 15 minutes. The calcium oxalate precipitate is used for the determination of calcium by the Kramer-Tisdall method.

For the determination of magnesium 5 cc. of the supernatant fluid (= 0.5 cc. of plasma) are pipetted off into a test-tube, 3.0 cc. of dispersing colloid added, then 1.0 cc. of dye solution and 1 cc. of 0.4 N NaOH. The contents are mixed thoroughly and compared in a colorimeter with Standard 2. The light used should be filtered through a color screen of 0.01 per cent dye solution. As a container for this we have used an oblong glass vessel about 25 cm. long, 15 cm. deep, and 1 to 2 cm. thick. The calculation is

(reading of standard/reading of unknown)  $\times 4$  = mg. of Mg per 100 cc. of plasma.

If the plasma magnesium is found or suspected to be too high to be determined in the range of the colorimeter, the mixture used for comparison with the standard must be diluted further, and the results obtained multiplied accordingly.

Thus, if the magnesium appears to be very high, 2 cc. of the mixture (containing plasma, dye, and alkali) used above may be diluted to 10 cc. with dispersing colloid, mixed well, and this mix-

TABLE I  
*Comparison with Standard of Known Magnesium Content*

Stand- ard Mg	Average by Kolthoff method	Error	Average by Briggs method	Average by Yoshimatsu method
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	1.01 $\pm$ 0.02 (0.03)	1.0	1.03 $\pm$ 0.02 (0.05)	
1	1.00 $\pm$ 0.01 (0.01)		1.02 $\pm$ 0.01 (0.02)	
1	0.99 $\pm$ 0.01 (0.01)	1.0	0.86 $\pm$ 0.01 (0.02)	
1	0.94 $\pm$ 0 (0.01)	6.0		0.87 $\pm$ 0 (0.01)
2	1.89 $\pm$ 0.01 (0.02)	5.5		1.77 $\pm$ 0.02 (0.03)
8	7.89 $\pm$ 0.01 (0.02)	1.38		
12	11.08 $\pm$ 0.12 (0.23)	2.66		
18	17.24 $\pm$ 0.05 (0.05)	4.25		

$\pm$  indicates the average deviation from the average result; the maximum deviation from the average result is given in parentheses.

ture compared with the same standard. The calculation is now (reading of standard/reading of unknown)  $\times 20$  = mg. of Mg per 100 cc. of plasma.

If the color is still too high, a third determination is made with 1 cc. of the first mixture (plasma, dye, and alkali) instead of 2 cc. and compared with the same standard. The calculation then is (reading of standard/reading of unknown)  $\times 40$  = mg. of Mg per 100 cc. of plasma. The results of the analyses are given in Table I.

The average error of our method is therefore 3.13 per cent Mg, varying from 2.5 per cent with the smaller amounts to 6.0 per cent for the larger amounts of magnesium. This error seems to be due to the decreased sensitiveness of color change with the depth



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of color produced by this amount of magnesium. The magnesium-dye color produced by 5 mg. of Mg per 100 cc. can be compared quite accurately with that produced by 10 mg. per 100 cc., and 10 mg. per 100 cc. can likewise be compared with 20 mg.; but

TABLE II  
*Analysis of Mixtures of Magnesium Chloride and Calcium Chloride*  
(Mg. per 100 Cc.)

Mg present	Mg found	Per cent error	Ca present	Ca found	Per cent error
0	0		2.0	1.98±0.05	0.75±0.25
2	1.97±0.01	1.5±0.2	10.0	9.95±0.05	0.5 ±0.5
5	4.89±0.06	2.2±1.2	15.0	15.05±0.05	0.35±0.35
20	19.6 ±0.2	2.0±1.0	20.0	19.97±0.1	0.15±0.5
0	0		30.0	30.15±0.15	0.5 ±1.0
30	29.2 ±0.2	2.3±0.7	30.0	30.2 ±0.3	0.7 ±1.0
50	44.6 ±0.45	10.7±0.9	30.0	32.5 ±0.3	8.1 ±1.0

TABLE III  
*Results Obtained on Rabbit Blood (Mg. of Magnesium per 100 Cc. Plasma)*

Average by our method	Average by Briggs method	Average by Yoshimatsu method
1.78 ± 0.05 (0.08)		1.86 ± 0.04 (0.06)
1.66 ± 0.06 (0.06)	1.17 ± 0.01 (0.01)	
2.25 ± 0.22 (0.23)	1.90 ± 0.26 (0.26)	
0.99 ± 0.03 (0.03)	0.91 ± 0.16 (0.16)	
1.02 ± 0	0.95 ± 0.06 (0.06)	
1.83 ± 0		1.905± 0.05 (0.10)
1.705± 0.05 (0.10)		1.865± 0.05 (0.10)
1.83 ± 0		1.80 ± 0
1.71		1.915± 0.13 (0.30)
1.72		1.85 ± 0.02 (0.20)
1.64		1.90 ± 0.09 (0.19)
1.75		1.89 ± 0

± indicates the average deviation from the average result; the maximum deviation from the average result is given in parentheses.

when 20 mg. standards are compared with 40 mg. solutions the error rises to 6.6 (33 per cent). With the higher concentrations of magnesium accurate results can be obtained by diluting in the manner described above.

Both calcium and magnesium can be determined accurately in mixtures of magnesium and calcium salts, provided the calcium is first removed by oxalation and determined by the Kramer and Tisdall method, and the magnesium then determined colorimetrically in the supernatant fluid, as shown in Table II.

Analyses of rabbit blood are given in Table III.

The results on a sample of rabbit blood to which known amounts of magnesium have been added are presented in Table IV.

TABLE IV  
*Amount of Magnesium Found in Rabbit Blood Sample, 2.10 Mg. of  
Magnesium per 100 Cc.*

Mg added	Amount found	Increment found	Error
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>
1	3.12 ± 1	1.02	2
2	4.01 ± 1	1.91	4.5
4	5.99 ± 0.09 (0.19)	3.89	3.0
6	7.55 ± 0.48 (0.48)	5.45	9.0
8	9.90 ± 0.03 (0.03)	7.80	2.5
16	17.57 ± 0.03 (0.03)	15.45	3.5

± indicates the average deviation from the average result; the maximum deviation from the average result is given in parentheses.

#### *Micromethod for Determination of Magnesium and Calcium*

The method which we have described above can be slightly modified so as to yield a satisfactory micromethod by which calcium and magnesium can be determined in 0.5 cc. of blood or in 0.1 cc. of blood plasma.

The reagents used for the magnesium determination are the same as those for the macrodetermination; but for the determination of calcium 0.001 N  $\text{KMnO}_4$  is used.

In this determination we use specially blown centrifuge tubes 7 mm. in diameter, 7 cm. long, and tapered toward the lower end at the same angle as the ordinary 15 cm. centrifuge tube. Our tubes were stoppered with ground glass stoppers, but this is not necessary. Before placing this tube into the ordinary centrifuge receptacle, a rubber stopper is placed in the bottom of the receptacle. This rubber stopper bears a circular depression in which the glass centrifuge tube rests and acts as a cushion. The barrel

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of the tube is surrounded by a second rubber stopper which fits in the metal receptacle and prevents wobbling during centrifugation.

0.5 cc. of blood is removed from a vein with a tuberculin syringe, placed in one of these centrifuge tubes which contains two or three small crystals of sodium citrate, stoppered, inverted two or three times, and centrifuged. 0.1 cc. of plasma is removed in a 1 cc. pipette graduated to 0.01 cc., transferred to another small centrifuge tube, 0.1 cc. of 3 per cent ammonium oxalate added, then 0.8 cc. of  $H_2O$  at 40–45°. The tube is stoppered, the contents mixed by inversion, placed in a water bath at 45° for half an hour, then centrifuged for 15 minutes.

For the determination of magnesium 0.5 cc. of the supernatant fluid is transferred to the microcolorimeter cup, 0.3 cc. of dispersing colloid added, then 0.1 cc. of dye solution and 0.1 cc. of 0.4 N NaOH. These are mixed thoroughly by stirring with a fine glass rod and compared with Standard 2 as for the macromethod. The calculation is the same as for the macromethod.

If the pink color due to the magnesium is too deep for the range of the colorimeter, 0.2 cc. of the mixture in the cup is pipetted off, diluted with 0.8 cc. of dispersing colloid, and this again compared with the standard. The results obtained by the above calculation, are, of course, multiplied by 5.

In order to determine the calcium, the oxalate precipitate in the centrifuge tube is washed three times with water, heated in a bath of boiling water, 0.5 cc. of N  $H_2SO_4$  added, and titrated with 0.001 N  $KMnO_4$  (1 cc. of  $KMnO_4$  = 0.02 mg. of Ca or 20 mg. per 100 cc. of plasma).

Comparison of the results obtained with the macro- and micro-method on six samples of known standard containing 2 mg. of Mg per 100 cc. of  $H_2O$  gave for the macromethod the average  $2.04 \pm 0.02$  (4); for the micromethod the average  $2.01 \pm 0.02$  (4).

In fifteen specimens of rabbit blood taken from eight different animals the results obtained with the micromethod showed an average difference of 0.02 mg. of Mg from those obtained with the macromethod; *i.e.*, less than 1.5 per cent difference. The maximum difference was 0.06 mg. of Mg. The micromethod is therefore practically as accurate as the macromethod.

*Colorimetric Determination of Magnesium in Urine*

Since the urine contains considerable amounts of ammonium salts as well as calcium salts, the phosphate ions as well as the calcium ions must be removed before the colorimetry in order to prevent the precipitation of ammonium magnesium phosphate. This is done by adding a 3.5 per cent solution of uranyl acetate drop by drop. The uranyl acetate removes hemoglobin and other blood and bile pigments from highly colored pathological urines.

The reagents used are otherwise the same as are used for the plasma macrodetermination.

TABLE V  
*Magnesium Content of Urine of Normal Human Beings*

Subject	Urine volume	Amount of Mg	
		Colorimetric	Gravimetric
	cc.	mg.	mg.
Da.....	740	211	201
Du.....	1110	201	193
Ba.....	830	238	235
Ha.....	875	241	247
Ge.....	900	213	210
Ol.....	875	235	233
An.....	925	217	210
Average difference $\pm$ 6 mg. ( $\pm$ 3 per cent)			
Maximum " $\pm$ 10 " ( $\pm$ 5 " " )			

5 cc. of urine are rendered just alkaline to phenol red with 1 drop of concentrated ammonium hydroxide, then just acid with 2 drops of 50 per cent HCl, 1.0 cc. of 1 per cent oxalic acid added, the mixture shaken vigorously, diluted with H<sub>2</sub>O to 10 cc., placed in a water bath at 85° for 5 minutes, again shaken vigorously, and centrifuged. The calcium oxalate can be used for the determination of calcium by the Kramer-Tisdall method.

For the determination of magnesium 2 cc. of the supernatant liquid are heated in a water bath to 90–100° and a 3.5 per cent solution of uranyl acetate added drop by drop until precipitation of the phosphates is just complete, as shown by the appearance of a brownish red precipitate when a sample drop is tested on a porcelain plate with 10 per cent potassium ferrocyanide. This usually

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requires about 1.0 cc. of uranyl acetate solution. The mixture is diluted to 10 cc. and centrifuged for 5 minutes. 1.0 cc. of the supernatant liquid is pipetted into a tube containing 7.0 cc. of dispersing colloid, 1 cc. of dye, and 1 cc. of 0.4 N NaOH added, mixed, and compared in the colorimeter with Standard 2.

The calculation is (reading of standard/reading of unknown)  $\times 20$  = mg. of Mg per 100 cc. of urine.

If the magnesium content is too high, 2 cc. of the mixture are taken, 8.0 cc. of dispersing colloid added, mixed, and compared with the same standard (Standard 2). The calculation is then (reading of standard/reading of unknown)  $\times 100$  = mg. of Mg per 100 cc. of urine.

The Mg content of the urine of normal human beings is given in Table V.

In urine samples from seven normal dogs whose excretion of magnesium ranged from 85 to 120 mg. of Mg during 24 hours, the average variation in the values obtained by the colorimetric and gravimetric methods was  $\pm 3$  mg. of Mg ( $\pm 3$  per cent), the maximal single difference was 5 mg. (6.6 per cent).

These observations indicate that the method is sufficiently accurate for clinical use.

### SUMMARY

1. The magnesium ions of the plasma can be determined colorimetrically with titan yellow or Clayton yellow in alkaline solution after the calcium has been precipitated out as oxalate.

2. This method avoids the necessity of precipitating the magnesium either as the phosphate ( $\text{MgNH}_4\text{PO}_4$ ) or as the oxyquinoline compound, and is therefore much simpler and much more rapid than the methods previously employed.

3. A micromethod is described for the accurate determination of magnesium and calcium in 0.1 cc. of plasma.

4. The colorimetric method can be used for determination of magnesium in urine, if the phosphates are first precipitated out with uranyl acetate.

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# **EFFECT OF RENAL INSUFFICIENCY UPON PLASMA MAGNESIUM AND MAGNESIUM EXCRETION AFTER INGESTION OF MAGNESIUM SULFATE\***

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Although epsom salt has been used as a cathartic since its introduction by Grew in 1675, little has been known regarding the amount of magnesium actually absorbed from the bowel and excreted by the kidneys; and the effect of renal insufficiency upon this process has been entirely overlooked. We have found in the literature only two determinations of magnesium in the urine after ingestion of epsom salt. Hay (1) found 28.1 per cent of the magnesium excreted in 24 hours after one normal individual ingested 20 gm. of epsom salt ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) and Yvon (2) found 21 per cent excreted after the same dose. In seven normal individuals we found, using the analytical methods described in the previous papers (3, 4), that 40 to 44 per cent (average 42.6 per cent) of the ingested Mg was excreted in the urine in 24 hours, while the plasma magnesium rose from an average concentration of 1.85 mg. of Mg per 100 cc. to an average concentration of 2.09 mg. (The greatest increase was 0.4 mg. of Mg per 100 cc.) In normal dogs and normal rabbits which received even larger doses (1 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo by stomach tube), the per cent of magnesium excreted and the rise of plasma magnesium were about the same. When doses of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ranging from 2 gm. to 10 gm. per kilo were given by stomach tube to eleven normal rabbits, the per cent of magnesium excreted remained quite constant re-

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ardless of dose, ranging from 35.8 to 52.3 per cent (average 42.4 per cent). Four normal dogs excreted  $42.6 \pm 3.7$  per cent.

In six normal rabbits doses of 2.0 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo by stomach tube caused only a very small rise of plasma magnesium, from  $2.03 \pm 0.14$  mg. per 100 cc. to  $3.01 \pm 1.27$  mg. After doses of 4 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo in two rabbits, plasma magnesium rose in less than 4 hours to  $4.04 \pm 0.89$  mg. per 100 cc.; after 6 gm. per kilo in two rabbits it rose to  $5.20 \pm 0.91$  mg.; after 8.0 gm. per kilo in three rabbits it rose to  $9.23 \pm 2.23$  mg.; in three rabbits after 10 gm. per kilo it rose to  $8.24 \pm 1.24$  mg.; and in one rabbit after 12 gm. per kilo it rose to 11.99 mg. In four normal dogs the effects were similar but slightly greater than with 2 gm. of  $\text{MgSO}_4$  per kilo, causing a rise of plasma Mg from 1.71 to 3.08 mg.; 3.0 gm., a rise to 4.7 mg.; 4.0 gm., a rise to 6.2 mg.; 6 gm., a rise from 2.4 to 10.7 mg.<sup>1</sup>

None of these normal animals went into coma.

However, when  $\text{MgSO}_4$  is given by mouth to nephrectomized animals or to animals whose kidneys have been injured by subcutaneous injection of  $\text{HgCl}_2$  or cantharides, the result is totally different (Fig. 1). After double nephrectomy the plasma magnesium in four rabbits spontaneously rose gradually to reach a level of  $16.8 \pm 0.8$  mg. per 100 cc. after 5 days. They then just reached the concentration at which coma developed.

When magnesium sulfate was given by stomach tube to nephrectomized rabbits 24 hours after the nephrectomy the plasma magnesium rose rapidly (within 3 to 4 hours) to much higher levels than in the normal rabbits, and coma developed whenever the level of 17 mg. per 100 cc. was reached.<sup>2</sup> The increases were as follows: In two rabbits after 2.0 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo by stomach tube there was a rise from  $3.13 \pm 0.76$  mg. to  $10.11 \pm 1.16$  mg.; in two rabbits after 2.5 gm. per kilo of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , a rise from  $3.38 \pm 0.49$  mg. to 13.75 and 17.89 respectively (the second

<sup>1</sup> Mg gluconate in doses equimolecular with 1 to 6.0 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo by stomach tube in normal rabbits or rabbits injected with  $\text{HgCl}_2$  did not raise plasma Mg as much as did  $\text{MgSO}_4$  (a maximum rise to 5.83 mg. of Mg per 100 cc. occurred in one rabbit injected with  $\text{HgCl}_2$ ).

<sup>2</sup> This coincides exactly with the concentration at which Neuwirth and Wallace (5) found that coma occurred in normal animals after subcutaneous injection of  $\text{MgSO}_4$ .

animal was in coma); in three rabbits after 3.0 gm. per kilo, a rise from  $3.08 \pm 0.63$  mg. to  $19.7 \pm 0.85$  mg. (coma); in two rabbits after 4.0 gm. per kilo, a rise from  $2.7 \pm 0.6$  mg. to 17.83 and 27.8 mg. respectively (both in coma); in one rabbit after 6 gm. per kilo, a rise from 2.76 mg. to 32.0 mg. (coma); and in one rabbit after 8

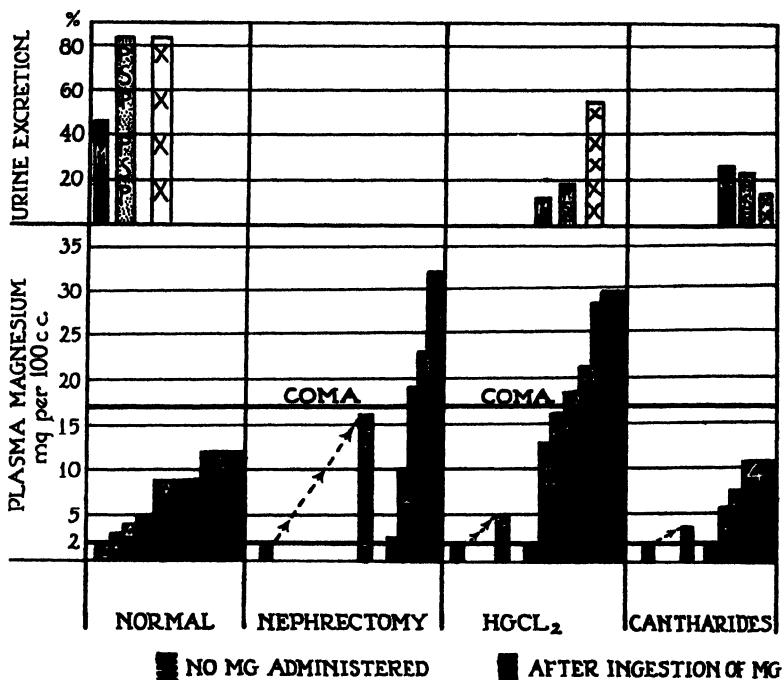


FIG. 1. Effect of renal insufficiency on plasma and urine magnesium. M represents per cent of ingested magnesium excreted in urine in 24 hours; PSP, per cent of injected phenolsulfonphthalein excreted in the urine; X, per cent of xylose excreted in the urine. The white figures in black areas indicate gm. of  $MgSO_4 \cdot 7H_2O$  per kilo by stomach tube which cause corresponding increases of plasma magnesium (average figures). Coma occurs in all the animals whose plasma Mg is above 17 mg. per 100 cc.

gm. per kilo, a rise from 2.17 mg. to 29.95 mg. (coma). Although in normal rabbits the Ca:Mg ratio is (3 - 4)/1, coma did not develop in the nephrectomized rabbits or other rabbits even when the Ca:Mg ratio fell to less than 1:1 unless the Mg was more than 16 mg. per 100 cc.

In six normal dogs after nephrectomy the plasma Mg gradually rose spontaneously during 4 to 6 days from a previous level of  $2.43 \pm 0.7$  mg. to from 9.47 to 20.9 mg. (average 15.4 mg.). Coma occurred in three dogs at 17 mg. of Mg or over. In nephrectomized dogs  $\text{MgSO}_4$  by stomach tube produced much greater increases in plasma magnesium than in normal dogs. The increases were as follows: In one nephrectomized dog after 2.5 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , per kilo, a rise from 2.15 mg. of Mg to 9.90 mg. in 3 hours; in three dogs after 3 gm. per kilo, a rise from  $2.94 \pm 0.2$  mg. to  $18.24 \pm 0.5$  mg. (all three in coma); in one dog after 4 gm. per kilo dose, a rise from 3.20 mg. to 20.7 mg. (coma); in one dog after 5.0 gm. per kilo, a rise from 3.0 mg. to 23.9 mg. (coma); in one dog after 6.0 gm. per kilo, a rise from 2.8 mg. to 26.87 mg. (coma); in one dog after 8.0 gm. per kilo, a rise from 3.47 mg. to 29.73 mg. (coma and rapid death).

In rabbits in which nephrosis with predominantly tubular injury was produced by subcutaneous injection of 5 to 15 mg. of  $\text{HgCl}_2$  per kilo, the conditions were similar to but less marked than those following nephrectomy. In eight rabbits which received no  $\text{MgSO}_4$  the plasma magnesium did not rise above  $4.87 \pm 1.53$  mg., to which level it rose gradually in 4 days. There was no evidence of coma. However, after the administration of  $\text{MgSO}_4$  by stomach tube, much less magnesium was excreted by these nephrotic kidneys, in which the tubules were injured more severely than the glomeruli, and coma was induced easily. Corresponding to the pathological findings, the excretion of phenolsulfonphthalein was reduced from a normal of  $86 \pm 5$  per cent to  $19.2 \pm 7$ ; i.e., to 22.3 per cent of the normal. The xylose excretion was reduced to a much less degree, from a normal of  $86.0 \pm 5.0$  per cent to  $66.1 \pm 5.0$  per cent (76.8 per cent of the normal). In these rabbits with nephrosis due to injection of  $\text{HgCl}_2$  the per cent of ingested magnesium excreted in the urine was  $11.8 \pm 2.2$  per cent (27.8 per cent of the normal). The ability to excrete magnesium and phenol-sulfonphthalein are thus reduced to an almost equal degree, while the excretion of xylose is much less reduced.

After the administration of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  by stomach tube to rabbits which had previously been rendered nephrotic by 10 mg. of  $\text{HgCl}_2$  per kilo, the following increases from previous levels of Mg per 100 cc. of plasma to maximum concentration reached 2 to

4 hours later were noted: in four rabbits after 2 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo (previous plasma Mg  $2.16 \pm 0.23$  mg.) a rise to  $14 \pm 2.2$  mg. (light coma or near coma) (two of these rabbits excreted  $10.87 \pm 1.24$  per cent of ingested Mg, i.e. 25.6 per cent of the normal excretion); in three rabbits after 2.5 to 2.75 gm. per kilo, a rise from  $2.00 \pm 0.09$  mg. to  $16.26 \pm 5.0$  mg. (coma); in three rabbits after 3.0 gm. per kilo, a rise from  $2.34 \pm 1.08$  mg. to  $18.2 \pm 0.3$  mg. (coma) ( $10.28 \pm 4.5$  per cent of ingested Mg was excreted in the urine, i.e. 20.4 per cent of normal excretion); in three rabbits after 4.0 gm. per kilo, a rise from  $2.07 \pm 0.11$  mg. to  $21.3 \pm 2.0$  mg. (coma); in one rabbit after 5.0 gm. per kilo, a rise from 2.07 mg. to 28.7 mg. (deep coma); in one rabbit after 6.0 gm. per kilo, a rise from 2.2 mg. to 29.8 mg. (deep coma).

In rabbits which were given 1 cc. of tincture of cantharides (= 0.6 mg. of cantharidin) per kilo subcutaneously, histological examination showed lesions of the glomeruli predominating over injury to the tubules. Corresponding to this, the phenolsulfonphthalein excretion fell to  $23.4 \pm 5.9$  per cent (27.2 per cent of normal), excretion of magnesium after ingestion fell only to  $28.7 \pm 2.4$  per cent (67.7 per cent of normal), and xylose excretion fell much more, to  $16.1 \pm 2.4$  per cent (18.7 per cent of normal). In four rabbits which received cantharides but no  $\text{MgSO}_4$  the plasma magnesium rose gradually during 3 days from a previous level of  $2.05 \pm 0.05$  mg. to  $3.86 \pm 0.24$  mg.

After the administration of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  by stomach tube to rabbits which had previously received 1 cc. per kilo of tincture of cantharides subcutaneously, the following increases in plasma were noted: In one rabbit after 2.0 gm. per kilo the plasma Mg rose to 5.9 mg.; in one rabbit after 2.5 gm. per kilo there was a rise from 2.27 mg. to 7.19 mg.; in three rabbits after 4.0 gm. per kilo, a rise from  $2.35 \pm 0.05$  mg. to  $12.2 \pm 2.4$  mg.; in one rabbit after 6.0 gm. per kilo, a rise from 1.86 mg. to 10.7 mg. None of these rabbits went into coma.

From these experiments it is evident that the plasma Mg rose less and the excretion of Mg in the urine was depressed less in animals injected with cantharides than in animals injected with  $\text{HgCl}_2$ .

Since the excretion of magnesium in the urine runs more nearly parallel to the excretion of phenolsulfonphthalein than to the ex-

cretion of xylose, it seems probable that most of the magnesium is excreted through the tubules. This corresponds well to the observations of Smith (6), Marshall (7), and Bieter (8) who have shown that magnesium is excreted by the aglomerular kidneys of the toadfish.

In animals in which coma was induced by increased plasma magnesium from the ingestion of  $\text{MgSO}_4$ , the coma could be terminated instantly and complete return to consciousness and motility induced by the intravenous injection of  $\text{CaCl}_2$ . These animals lived longer than those which had not received  $\text{CaCl}_2$  but not as long as those which had not received  $\text{MgSO}_4$ .

Administration of  $\text{Na}_2\text{SO}_4$  in doses equimolecular with 1 to 12 gm. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo never brought on coma in normal animals or animals injected with  $\text{HgCl}_2$ . Sodium sulfate should therefore be the saline cathartic of choice in nephritic patients.

#### SUMMARY AND CONCLUSIONS

1. Normal animals and normal human beings excrete about 42 per cent of ingested magnesium in the urine in 24 hours. The per cent of magnesium excreted is fairly constant and is independent both of the dose and of the concentration of magnesium in the plasma.

2. Since the magnesium excretion is depressed by injury of the tubules more than by injury of the glomeruli and follows the phenolsulfonphthalein excretion curve but not the xylose excretion curve, most of the magnesium is excreted through the renal tubules.

3. In normal animals and normal human beings ordinary doses of ingested  $\text{MgSO}_4$  scarcely raise the plasma magnesium.

4. In nephrectomized animals or in animals with injured kidneys, ingestion of  $\text{MgSO}_4$  causes a rapid and intense increase in plasma magnesium, and may induce coma.

5. On account of this increase in plasma magnesium, it is probable that many clinical cases of coma in nephritic individuals, supposed to be uremic coma, are produced by the use of magnesium salts as purgatives. These patients could probably be resuscitated from this coma by the intravenous injection of calcium salts.

6. Sodium sulfate should be the saline of choice in patients with renal insufficiency.

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## PROTEIN FRACTIONS OF THE HUMAN STRAIN (H-37) OF TUBERCLE BACILLUS

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The isolation of protein material from the tubercle bacillus has been accomplished by a number of workers, and their findings have been reviewed by the writers in a preliminary article (1) and by Gough in a more recent paper (2).

It had been found in this laboratory that fractionation of the proteins of a scarlatinal strain of *Streptococcus hæmolyticus* was greatly facilitated by an initial acidification of the pulverized, defatted microorganisms and by the avoidance of alkali until all protein soluble in neutral buffer had been removed (3). A preliminary study of this method with a non-pathogenic member of the acid-fast group, the timothy-grass bacillus, *Mycobacterium phlei*, showed its applicability (4), and the method was accordingly used for the fractionation of the proteins of the human Strain H-37 of the tubercle bacillus (1). The present communication records the details of the experiments and describes the results of further work. A number of fractions have been obtained, at least two of which are distinct and different antigens. All of the fractions tested showed a high tuberculin activity, in contradistinction to those isolated by Gough (2), and, with one minor exception, appeared to be free from specific carbohydrate.

### EXPERIMENTAL

The tubercle bacilli (Strain H-37) were grown by Mr. John Glenn in the H. K. Mulford Biological Laboratories of Sharp and

\* National Tuberculosis Association Fellow. The work described in this communication was made possible by the National Tuberculosis Association and the Harkness Research Fund of the Presbyterian Hospital.



Dohme, Glenolden, Pennsylvania, through the courtesy of Dr. John Reichel. Two types of material were used, both originating from 3 week-old cultures grown on Long's synthetic medium: (a) frozen and vacuum-dried organisms, for Preparations 701 and 702; and (b) cells which had been killed by immersion in acetate buffer at pH 4.0 and storage in the cold, with frequent shaking, for at least 30 days (Preparations 703 and 704). The writers wish to thank Dr. Reichel and Mr. Glenn most heartily for their generous assistance and also for the Anti-strain H-37 horse serum used in this investigation.

The frozen and dried bacilli were extracted four times in the cold for about 2 hours with redistilled acetone; they were centrifuged in the refrigerating centrifuge<sup>1</sup> between extractions and the last acetone extraction was allowed to stand overnight in the ice box. The bacilli were then similarly extracted three times with purified ether.<sup>2</sup> During the extractions the organisms were stirred frequently, and were finally dried *in vacuo*. The buffer-killed bacilli were worked up in the same way after an initial centrifugation from the buffer solution. The dried bacilli were placed in a ball mill and ground for about 10 days, until intact cells could no longer be observed in stained smears. The ground bacilli were transferred from the ball mill into centrifuge bottles with the aid of redistilled acetone. After centrifuging and pouring off the acetone two additional ether extractions were carried out in the cold and the organisms were finally dried *in vacuo*.

In Table I are given the quantities of bacilli used and the volumes of the extraction liquids.

The cell residues were stirred for about 6 hours in the cold successively with 0.2 N acetate buffer at pH 4.0 (Fraction C),<sup>3</sup> with M/15 phosphate buffer at pH 6.5 (Fraction D), with water containing enough ammonia to keep the pH at 8.3 to 8.5 (Fraction E), with water made alkaline with 0.1 N sodium hydroxide

<sup>1</sup> Manufactured by the International Equipment Company, Boston. All centrifugations were carried out in this way, in order to minimize denaturation and, in the later processes, enzyme action.

<sup>2</sup> The ether was washed well with water and dried over calcium chloride and stick sodium hydroxide.

<sup>3</sup> This fraction contained specific polysaccharides and practically no protein.

to about pH 9.0 (Fraction F), and with water made alkaline to about pH 11.0 (Fraction G). The residual material was stirred or shaken in turn with 0.1, 0.2, and 0.5 *N* sodium hydroxide solution at room temperature (Fractions K, K', and K'').

Each extraction was poured off after centrifuging in the cold and treated in the case of the D and K fractions with 50 per cent or glacial acetic acid, in the case of the other fractions with *N* acetic acid to maximum flocculation. Each fraction was centrifuged off (in the cold), suspended in about 400 cc. of chilled water, and re-dissolved as follows: the D fractions with the aid of chilled sodium bicarbonate solution neutralized to pH 7.2 to 7.4 by cautious addi-

TABLE I  
*Amounts of Tubercle Bacilli and Extraction Liquids Used*

	Prepara- tion 701	Prepara- tion 702	Prepara- tion 703	Prepara- tion 704
Weight before extraction, gm.....	16	22	50*	100*
Volumes of acetone and ether for each extraction, cc.....	200	400	200	500†
Weight after defatting and grinding, gm.....	9.3	16.0	7.5	16.5
Volumes of aqueous solution for each extraction, liters.....	3	3	2	3

\* Approximate moist weight.

† In this preparation the aqueous buffer solution was siphoned off instead of being centrifuged. Five acetone extractions were made, followed by one with 250 cc. of ether.

tion of glacial acetic acid; the E fractions with 0.1 *N* aqueous ammonia added drop by drop to pH 8.3 to 8.5; the F, G, and K fractions by dropwise addition of *N* sodium hydroxide until solution took place, care being taken that the pH did not exceed 9.0 in the case of Fraction F and 11.0 for the others.

The resulting turbid solutions were centrifuged (always in the cold) and the supernatant liquids were precipitated as above. This procedure was repeated two or three times more with reduction of the volume of solution in the case of the smaller fractions. The solutions were then run through Berkefeld V filters,<sup>4</sup> the process

<sup>4</sup> The Berkefeld filters were first washed in the case of the D fractions with neutralized sodium bicarbonate, in the case of the other fractions with dilute alkali followed by water.

being repeated or N filters being used if the filtrates were not clear. The filtrates were precipitated as before and centrifuged. The products were washed three times with water acidified with a few drops of N acetic acid, centrifuged between washings, and then washed three times with redistilled acetone. After filtering on a Buchner funnel with the aid of this solvent the fractions were dried *in vacuo* over paraffin, calcium chloride, and sodium hydroxide pellets. For analysis portions were dried to constant weight in a high vacuum over phosphorus pentoxide.

In Preparation 702 Fraction L consisted of the insoluble material collected in the centrifugation and filtration of Fractions D, E, and G, taken up in 250 cc. of 0.1 N sodium hydroxide, run through a Berkefeld filter, and isolated after acidification. The filter residues were combined with the insoluble material collected from the other fractions, dissolved in 500 cc. of 0.5 N sodium hydroxide, and isolated as Fraction L'.

After each precipitation the pH of the supernatant liquid was determined by the bicolor standard method (5). The supernatant liquids contained appreciable amounts of nitrogen but yielded very little protein on saturation with ammonium sulfate and were discarded after testing. A rough idea of the points of minimum solubility of the fractions may be obtained from the pH of the supernatant liquids. For the different fractions these varied between the following limits, depending somewhat on the state of purity of the product: Fraction D, 3.4 to 4.2; Fraction E, 3.6 to 4.5; Fraction F, 4.0 to 4.5; Fraction G, 3.7 to 4.7; Fraction K, 4.5 to 5.0. The general trend was, as with *Streptococcus hæmolyticus* (3), toward less acid products as the alkalinity of the extraction medium was increased.

The properties of the fractions are summarized in Table II. In Preparations 702 and 703 no F fraction was isolated, so that this material was contained in the G fraction. In Preparation 704 Fractions K' and K'' were omitted.

For the final solutions weighed amounts of the substances were dissolved in water in the case of the D fractions with the aid of neutralized sodium bicarbonate; in the case of the other fractions with the aid of 0.1 N sodium hydroxide, phenolphthalein being used as indicator. When only traces of insoluble material remained, a drop or two of very dilute acetic acid was added to discharge the color of the indicator. The pH of the resulting solutions was

TABLE II  
*Properties of Tubercle Bacillus Protein Fractions*

Preparation No.	Fraction	Yield	$[\alpha]_D$	pH*	Nitrogen	Phosphorus	Basic ash
		gm.	degrees		per cent	per cent	per cent
701	D	0.23	+9	6.9	15.7	3.4	0.7
	E	0.20	+11	6.4	15.4	2.0	0.5
	F	0.09	-25	6.3	15.0	1.8	1.4
	G	0.02	-31	6.1			
	K	0.56	-61	6.3	16.2	0.4	0.4
	K'	0.35	-50	6.4	14.9	0.04	0.3
	K''	0.07	-18	7.3	11.2		1.9
Total yield....		1.52(16.3%)†					
702	D	0.23	+9	6.4	15.9	3.7	0.8
	E	0.35	-19	6.8	15.8	2.1	0.2
	G	0.24	-28	6.3	15.7	2.1	0.2
	K	1.66	-56	6.2	15.3	0.6	0.3
	K'	0.49	-51	6.4	15.3	0.06	0.2
	K''	0.02	-46	5.8			
	L	0.07	-39	5.9	15.2	0.11	0.5
	L'	0.25	-65	6.1	10.0		
Total yield....		3.31(20.7%)†					
703	D‡	0.19	-57	6.3	14.3	5.5	1.7
	E	0.12	-21	6.1	14.7	2.3	0.4
	G	0.32	-14	5.3	16.0	2.8	0.3
	K	1.06	-48	6.3	16.0	0.9	0.1
	K'	0.06					
	K''	0.03					
Total yield....		1.78(23.7%)†					
704	D	0.67	-57	6.6	14.5	5.0	2.4
	E	0.60	-39	6.0	16.1	2.2	0.3
	F	0.19	-46	6.3	15.9	1.4	0.3
	G	0.42	-46	6.1	15.4	1.5	0.5
	K	1.55	-53	6.5	15.8	0.6	0.3
Total yield....		3.43(20.8%)†					

\* pH at which optical rotation was determined.

† Of the dried defatted cells. The alkaline extraction residue from Preparation 702 weighed 1.34 gm.

‡ The turbid supernatant liquids from the final aqueous washing and the first acetone washing of the D fraction yielded an additional 0.05 gm.; the washings from the K fraction an additional 0.07 gm.

usually from 6.0 to 7.0 and the fractions remained in solution in this range.

TABLE III  
*Precipitin Reactions of Tubercle Bacillus Fractions with Antisera to the D and K Fractions*

	Preparation	Anti-D serum		Anti-K serum	
		Rabbit 7.35	Rabbit 7.38	Rabbit 1.48	Rabbit 1.47
Human	701-D	+±(+++)		±(+)	-(-)
	702-D	++(++±)	+(++±)	±(++±)	
	703-D	+(++)		-(-)	
	704-D	+(+±)		-(-)	
	701-E	++(++++)		+(++±)	
	702-E	+±(++)		+(++)	
	703-E		+(++)		+(+±)
	704-E	+(+)		+(+)	
	704-F	+(++)		+(++)	
	702-G	+±(++±)		+±(++++)	
	703-G		+(+±)		-(+)
	704-G	+(+±)		+±(++±)	
	701-K	±(+)		++(++++)	+(++±)
	702-K	±(++)		++(++++)	
	703-K		-(±)		+(+++)
	704-K	-(±)		+±(++±)	
	519-B	-(-)	-(-)	-(+)	-(-)
	519-C	-(-)	-(-)	-(-)	-(-)
Timothy	602-D	-(-)		-(-)	
	602-E	±(+)		+(++)	
	601-F	±(±)		±(±)	
	601-G	±(+)		+±(++±)	
	601-K	-(±)		++(++++)	

The D fractions were used at a dilution of 1:4000 by weight, as were Preparations 703-E and 704-F; Preparations 701-E, 702-E, 702-G, 602-E, 601-F, and 601-G at a dilution of 1:3000; the others at a dilution of 1:2000. Preparations 601 and 602 were timothy-grass bacillus fractions (4). The readings in parentheses were taken after centrifugation. Preparations 519-B and 519-C were the low and high rotating specific polysaccharides of Strain H-37 (10) at a dilution of 1:200,000.

The ash determinations were carried out after treatment with sulfuric and nitric acids, the ash being weighed as calcium sulfate and calculated as calcium. In all cases in which these determinations were made the values for the optical rotation, nitrogen, and phos-

phorus in Table II were calculated to the ash-free basis. Nitrogen was determined by the micro-Kjeldahl method; phosphorus by the Pregl-Lieb method (6).

*Serological Tests*—In the production of the antisera, Rabbits 1.47 and 1.48 were given three to four intravenous injections per week of a 0.1 per cent solution of Preparation 701-K; 0.5 cc. was injected at the beginning and the dose increased toward the end to 2 cc. A total of 29 mg. was injected into each rabbit over a period of 7 weeks. Rabbits 7.35, 7.36, and 7.38 were similarly injected with 21 mg. of Preparation 702-D. The sera were taken 6 days after the last injection. All but the serum from Rabbit 7.36 contained precipitin in appreciable amounts.

The precipitin tests were carried out on 0.3 cc. portions of serum and the protein dilutions, together with the usual controls. The contents of the tubes were mixed and allowed to stand in the water bath at 37° for 2 hours and overnight in the ice box. The readings in parentheses were taken after centrifugation for 5 minutes at about 1000 revolutions per minute. Several dilutions of the protein fractions were used, but only that giving the heaviest precipitation is recorded in Table III. The controls are also omitted.

#### DISCUSSION

The data summarized in Table II show sufficient variation in the properties of the single fractions to justify the conclusion that none has yet been obtained as a definite chemical individual. Moreover, the differences between the data for the D fractions prepared from the frozen and dried organisms (Preparations 701 and 702) and those obtained from the buffer-killed bacilli (Preparations 703 and 704) indicate that the initial treatment of the cells may influence the proportions of the components entering into each fraction. These differences are not sufficient, however, to mask the antigenic distinction between the D and K fractions discussed below—a difference which was evident in all preparations. A further regularity is that also observed in the proteins of the strains of *Streptococcus haemolyticus* (3) and timothy-grass bacillus (4) studied; namely, the decrease in the phosphorus content of the fractions with increase in the alkalinity of the successive extraction media. The apparent exception of Fraction G may be due to the similarity of Fractions E, F, and G, so that the fractions

could be arranged as follows in the order of decreasing phosphorus content:  $D > E, F, G > K > K'$ .

With the exception of the last, the fractions contain enough phosphorus to permit their tentative classification as nucleoproteins by analogy with those of other microorganisms (*cf.* also (3)). It will be noted that Fraction D corresponds roughly to the water-soluble protein of Johnson (7), Coghill (8), and Gough (2), while the other fractions would be included in the alkali-soluble protein of the first two workers. However, in the present studies, all operations have been carried out in the cold to prevent, as far as possible, secondary changes due to the active enzymes present in bacterial cells. Also, by the use of low temperatures and short exposure to non-aqueous solvents it has been sought to avoid denaturation of the proteins. The writers therefore believe that their fractions are more nearly related to the actual cell constituents than those isolated by the aforementioned workers. The relation of the fractions to those reported by Levene (9) will be studied.

Precipitin reactions of the fractions against an antibacterial (Strain H-37) horse serum are given in (1) and are not repeated, since the serum was very weak in antiprotein. The weak reactions are, however, an indication that the protein fractions were relatively free from specific carbohydrates, since the serum gave strong antipolysaccharide reactions. Thus only the very minor Fraction K' agreed in this respect with Gough's "albumin" fraction (2).

In Table III are given the precipitin reactions of the fractions with antisera prepared by injecting rabbits with the D and K fractions of the human (H-37) strain. With one trifling exception these sera were free from antibodies to the two specific polysaccharides isolated from the same strain (10). It will be noted that the D fractions react best in the D antisera and the K fractions precipitate the anti-K sera most strongly, while the cross reactions of Fraction D in the anti-K sera and Fraction K in the anti-D sera are almost negligible, particularly in the case of Preparations 703 and 704, in which the writers believe they have obtained the most complete fractionation. Thus at least two separate and distinct cellular protein antigens may be isolated from the defatted cells of the human Strain H-37 of tubercle bacillus. The serological tests on these fractions are thus in accord, in so far as a comparison is possible, with Coghill's belief (8) that the water-

soluble and alkali-soluble proteins were different. This rested mainly on the differences in the basic nitrogen and its distribution.

As for the E, F, and G fractions, it will be observed that they reacted in both the anti-D and the anti-K sera. Whether this is due to the presence of these fractions in the D and K used for the injections, or whether these fractions consist of D and K in a less easily separable form cannot be stated at present.

Table III also shows the reactions of these sera with the corresponding timothy-grass bacillus fractions (4). It will be noted that Fraction D failed to react in either anti-human D or K serum,

TABLE IV

*Absorption of H-37 Antiprotein Sera with Timothy-Grass Bacillus Fractions*

Absorbed with.....	Anti-D serum		Anti-K serum	
	Rabbit 7.35		Rabbit 1.48	
	Preparation 602-E	Preparation 601-K	Preparation 602-E	Preparation 601-K
Set up against				
Preparation 702-D	+(+±)	+(+)	-(-)	-(-)
“ 702-E	-(±)	-(±)	-(-)	-(-)
“ 701-F	+(+)	-(±)	-(±)	-(-)
“ 701-G	±(+)	-(±)	±(+)	-(-)
“ 702-K	-(-)	-(-)		-(-)
“ 702-K'		-(-)		
“ 702-K''		-(-)		
“ 602-E	-(-)		-(-)	
“ 601-K		±(±)		- (±)

while the others reacted only very weakly in the anti-D serum and definitely (except F) in the anti-K serum. The apparent type specificity of the D fraction was tested further by absorbing sera from Rabbits 7.35 and 1.48 with small amounts of 1:1000 timothy Fractions E and K, until a further addition, after 2 hours at 37° and overnight in the ice box, failed to yield a precipitate. The supernatant liquids were then set up against the human fractions indicated (Table IV). It will be seen that the timothy E and K fractions failed to remove the human Fraction D antibodies, but removed those for the other fractions (except F). Tests of the D fraction against anti-bovine and anti-avian strain rabbit sera, how-



ever, indicated a closer relationship, so that no actual type specificity can be claimed for Fraction D.

There is thus some evidence that Fraction D of the human strain shows no antigenic relation to the proteins of the timothy-grass bacillus, whereas the other fractions (except F) do. However, Fraction D of the timothy-grass bacillus precipitates the anti-bacterial Strain H-37 horse serum, although it does not precipitate the anti-protein sera. Since the cross reaction in the first instance might have been due to non-specific polysaccharide in the timothy Fraction D, two rabbits were injected repeatedly with this fraction, but both failed to yield precipitins. It is hoped to obtain further evidence on this point at a later date, as well as on the apparent failure of the F fractions to show cross reactions.

Tests by Dr. Sabin and Dr. Smithburn<sup>a</sup> of The Rockefeller Institute for Medical Research show that the protein fractions of Strain H-37 possess a tuberculin activity comparable with that of the MA 100 standard tuberculin used for comparison. The D fraction seemed especially potent, in marked contrast to the low activity reported for the water-soluble protein by Gough (2). The activity of the other fractions contrasts with the failure of the alkali-soluble protein to react, as reported by Coghill (8).

The writers have previously isolated two specific polysaccharide fractions from Strain H-37 of tubercle bacillus and have shown that these react with independent antibodies in an antibacterial Strain H-37 horse serum (10). Granted that the products isolated represent actual cell components, there must now be added to the minimum of two complex carbohydrate antigens thus indicated in the defatted tubercle bacillus cell at least two additional protein antigens which appear to belong to the type of the nucleoproteins.<sup>b</sup> There would thus seem to be at least four separable, distinct antigenic components to the defatted human type tubercle bacillus cell, and there is no reason to suppose that the limit to the complexity of the protoplasm of the microorganism has yet

<sup>a</sup> Unpublished results.

<sup>b</sup> Fractions E, F, and G yielded precipitates in a weak rabbit serum obtained by injecting a formalized suspension of Strain H-37. The serum did not precipitate Fractions D and K, so that the presence of a third protein antigen in Fractions E, F, and G may be indicated by this preliminary experiment.

been approached. Further efforts are being made to obtain approximations to definite, constant protein fractions. In the meantime, much additional work has been done on the carbohydrates and this will be reported in a separate communication.

#### SUMMARY

1. A method is given for the fractionation of the proteins of the defatted human (H-37) strain of tubercle bacillus under conditions designed to minimize enzyme action and denaturation and to effect a separation from the specific polysaccharides.

2. The physical, chemical, and immunological properties of the fractions so obtained are given.

3. It is shown that at least two of the fractions represent separate and distinct antigens, making a total of four so far indicated in the defatted bacillary cell.

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# THE REFRACTIVITY OF HEAT-DENATURED EGG ALBUMIN

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There is at present no general agreement among investigators as to whether the alterations of a protein, constituting denaturation, are purely physical, *i.e.* involve only a change in the state of aggregation, or are fundamentally chemical in the sense that there occurs an internal structural rearrangement of the molecule which results in secondary changes in physical properties. One of the most certain indications of structural alteration in a chemical compound is a change in its molecular refractivity. The refractivities of native and heat-denatured egg albumin have therefore been studied in order to discover if structural alteration actually does accompany denaturation. The specific refractive increment of crystallizable egg albumin has also been determined with greater accuracy than previously given by Haas (1).

## EXPERIMENTAL

The egg albumin was prepared by the method of Sørensen and Høyrup (2) and was dialyzed for 28 days against frequent changes of distilled water. A 1 per cent solution showed specific conductivity  $6.75 \times 10^{-5}$  mhos at 25° and pH 4.92. Protein concentration was determined gravimetrically by the method of Young (3).

The measurements comprise two series. In the first series a Zeiss dipping refractometer was used to obtain absolute measurements of refractive index; in the second series the change of refractive index accompanying heat denaturation was determined with a Zeiss interferometer.

*First Series*—The specific refractive increment  $\alpha$ , defined according to the equation,  $\eta_{\text{solution}} - \eta_{\text{solvent}} = \alpha C$ , where  $\eta$  is the refractive index and  $C$  is the protein concentration in gm. per 100 cc.,

was determined for native and heat-denatured egg albumin with a dipping refractometer with auxiliary prism.

Since heat-denatured egg albumin forms a clear solution only above pH 7 on the alkaline side of the isoelectric point, a considerable quantity of sodium hydroxide had to be added to our stock solutions. In calculating the specific refractive increment the approximate assumption was made that the refractivity of the

TABLE I  
*Specific Refractive Increment of Egg Albumin*

Native egg albumin				Heat-denatured egg albumin			
Gm. per 100 cc. solution	$\alpha$	Gm. per 100 cc. solution	$\alpha$	Gm. per 100 cc. solution	$\alpha$	Gm. per 100 cc. solution	$\alpha$
4.23	0.00188	6.04	0.00186	3.99	0.00189	3.76	0.00181
3.36	0.00187	4.99	0.00185	2.92	0.00188	3.76	0.00183
2.48	0.00186	4.08	0.00185	1.915	0.001865	3.71	0.00183
1.37	0.00189	3.11	0.00185	0.646	0.00183	3.74	0.00187
0.744	0.00184	2.02	0.00187	6.64	0.00182	3.71	0.001855
6.61	0.00184	1.05	0.00189	5.72	0.00181	3.70	0.00186
5.76	0.00185	7.05	0.00182	4.77	0.00181	3.74	0.00185
4.78	0.00184	6.01	0.001855	3.94	0.00181	3.71	0.00185
3.91	0.00186	5.04	0.00184	3.01	0.00180	3.64	0.00186
2.95	0.00185	3.99	0.00184	2.08	0.00181	3.77	0.00189
1.91	0.00186	3.09	0.00181	5.46	0.00187	3.72	(0.00191)
0.949	(0.00193)	2.16	(0.00179)	5.50	0.00188	3.76	0.00184
7.04	0.00187			3.77	(0.00192)	3.72	0.00188
				3.77	0.001875	3.73	0.00183
				3.70	0.001835		
Mean* = 0.001854 $\pm$ 0.000014				Mean* = 0.001844 $\pm$ 0.000026			
				" of all measurements* =			
				0.00185 $\pm$ 0.00002			

\* The values in parentheses are omitted in the calculation of the mean.

added sodium hydroxide is the same as in a pure sodium hydroxide solution of the same analytical sodium concentration.

Table I summarizes the measurements. The pH of the unheated solutions was varied from 4.6 to 5.8 but no drift of the  $\alpha$  values was observed as a function of pH. The same is true of the values for the heat-denatured egg albumin solutions of pH 6.9 to 7.4.

It will be seen from Table I that the specific refractive increments of native and alkaline heat-denatured egg albumin are identical within the limits of experimental error of the method used. It therefore seems desirable to combine all the data to give the single value,  $\alpha = 0.00185 \pm 0.00002$ , for the mean specific refractive increment of egg albumin.

*Second Series*—Since measurements with a dipping refractometer reading to only six significant figures are not sufficiently accurate to detect any change in refractivity accompanying heat denaturation, a second series of very accurate measurements of relative refractivity was carried out with a Zeiss interferometer reading to two further decimals.

TABLE II  
*Change of Refractivity Accompanying Heat Denaturation*

Experiment No . . . . .	1	2	3	4
Egg albumin per 100 cc. solution, gm.....	4.77	1.76	3.79	4.04
pH before heating.....	8.16	6.59	8.98	
Observed reading.....	+256.0	+87.7	+186.3	+205.8
Blank correction.....	-11.6	-11.6	-11.6	-11.6
Corrected reading.....	+244.4	+76.1	+174.7	+194.2
“ “ for 1 per cent solution.....	+51.2	+43.3	+46.1	+48.1
$\Delta\eta^{18^\circ}$ for 1 per cent solution $\times 10^3$ .....	+23.8	+20.1	+21.4	+22.4

Mean  $\Delta\eta = 21.9 \times 10^{-3}$

The method was to add sufficient sodium hydroxide to the egg albumin stock solutions to keep them perfectly clear on heating. The sample was thoroughly mixed and then divided into three portions. One portion was heated at 90–95° for 5 minutes in a water bath. The heated container was filled completely and stoppered so as to leave no air space into which evaporation might occur. Also foaming and bubbles which could give rise to concentration changes due to the separation of adsorbed films were avoided by heating to temperatures below 100°. The other two portions remained unheated. The solutions were placed in 4 cm. interferometer cells provided with glass stirrers to facilitate the

establishment of temperature equality. First, both interferometer cells were filled with the unheated solutions. This gave the zero reading. Then the heated solution was placed in one cell. Successive readings with the heated and unheated solution alternately in one interferometer cell gave good agreement ( $\pm 0.2$  scale division). Table II gives the data. The blank correction is the mean of four determinations carried out with a sodium hydroxide solution in place of the sodium hydroxide-containing egg albumin solutions.

The change of molecular refractivity ( $\Delta M$ ) may be at least approximately estimated from the observed change of refractive index ( $\Delta \eta$ ) by using the Gladstone-Dale formula for the specific refractivity of solutions.

$$R = \frac{1}{p} \frac{\eta_{\text{solution}} - 1}{d_{\text{solution}}} \times 100 - \frac{1}{p} \frac{\eta_{\text{H}_2\text{O}} - 1}{d_{\text{H}_2\text{O}}} (100 - p)$$

where  $R$  is the specific refractivity of the solute;  $p$  is the weight per cent of solute;  $\eta$ , the refractive index; and  $d$ , the density. The change of molecular refractivity is  $\Delta M = m\Delta R = m(R_d - R_n) = +74.5$  for the sodium D line.  $m$  is the molecular weight. The subscripts refer to native ( $n$ ) and heat-denatured ( $d$ ) egg albumin. In making the calculation it is assumed that the density of the native and denatured egg albumin solutions are identical ( $d = 1.00126$ ). This assumption is supported by the observation of Loughlin and Lewis (4) that no volume change accompanies denaturation. Also, in agreement with the work of Burk and Greenberg (5) and Huang and Wu (6), the molecular weights of the two modifications are taken to be identical and equal to 34,000.

#### DISCUSSION

The experimentally observed change in the refractivity of egg albumin accompanying heat denaturation must be regarded as strong evidence in favor of the chemical interpretation of the formation of denatured proteins. Indeed, the bulk of the available evidence tends to confirm this hypothesis. Nine properties of egg albumin are known to be altered by heat denaturation: (1) Denatured egg albumin is by definition completely insoluble in the region of its isoelectric point, particularly in the presence of neutral salts. This is in contrast to the extreme solubility of native egg

albumin in dilute salt solutions. (2) The insolubility of denatured egg albumin as compared with native egg albumin is an expression of the greater affinity of the molecules of the former for each other and their lesser affinity for water. Another evidence of the same thing is the lesser degree of hydration of denatured egg albumin as demonstrated by Sørensen and Sørensen (7), Katz (8), and Barker (9). (3) The viscosity of egg albumin solutions is increased by denaturation (Loughlin and Lewis (4)). The extent of the increase depends upon protein concentration, pH, and other factors. Under certain conditions stiff gels may be formed (Hopkins (10), Anson and Mirsky (11)). (4) Harris (12) and Pauli and Weiss (13) have shown that various modifications of egg albumin develop a reddish color in the presence of sodium nitroferrocyanide in alkaline solution. This is interpreted as indicating the formation of free —SH groups in the molecule. (5) The work of Herlitzka (14) and our own measurements indicate a slight but definite increase in refractivity accompanying the heating of egg albumin and its precipitation by heavy metal salts. (6) The specific optical rotation is in general increased (in a negative sense) by denaturation (Holden and Freeman (15)). Barker (16) found the rotatory power to have a definite and reproducible value under definite conditions. (7) Bizarro (17) and Mastin and Schryver (18) have demonstrated that heating increases the ease of digestibility by pepsin and trypsin. (8) Broeck (19) and Wu, Broeck, and Li (20) have studied the antigenic properties of egg albumin and have shown that both the precipitin and anaphylactic reactions are altered by denaturation. (9) The ultraviolet absorption spectra of native and of strongly acid and alkaline egg albumin (sufficient to cause denaturation) are very similar in form, but the absorption is greater at a given wave-length for the later than for the former. Our own unpublished observations in collaboration with J. Burnham indicate the same to be true for heated egg albumin.

Two or three properties are not altered measurably in denaturation. This was shown for cataphoretic mobility by Abramson (21) and for molecular weight in urea solution by Burk and Greenberg (5) and Huang and Wu (6). A change in acid-base-combining power is still a matter of dispute. Hendrix and Wilson (22) and Barker (16) observed in general a marked change in acid-



base-combining power to accompany heat denaturation. Booth (23), however, found the combining curves to be identical within the limits of experimental error.

This brief summary of the literature shows that all of the properties which would be expected to be most sensitive to structural changes in the egg albumin molecule (in particular the optical properties) actually are altered. The unaltered properties are those which should be less sensitive to structural rearrangement.

It is perhaps useless in the present state of knowledge to speculate as to the chemical alteration of the molecule responsible for the observed change in refractive index, since many types of alteration could lead to the same result. However, for convenience one may in a purely formal way express the equivalence of the observed change in terms of one particular possible alteration of structure as, for example, that corresponding to the keto-enol transformation,  $\Delta M_D = +1.7$ . Thus the observed increase in molecular refractivity of egg albumin during heat denaturation is equivalent to the keto-enol change occurring in approximately forty-three groups. It should be emphasized, however, that there exists no experimental basis for supposing that a keto-enol change actually accounts for the observed increase. The comparison is made simply as an aid to appreciating the magnitude of the observed alteration in molecular refractivity.

The value which we have found for the specific refractive increment differs appreciably from that due to Haas (1) and given in the International Critical Tables (24). Haas used a Pulfrich refractometer and obtained the value  $0.00177 \pm 0.00006$ , which was the same within experimental accuracy in dialyzed and in salt- and alkali-containing egg albumin solutions. This value seems to us to be less accurate than our own,  $0.00185 \pm 0.00002$ , because it was determined with much more dilute solutions (1.4 per cent as compared to about 4 per cent), because the number of measurements was fewer (twenty as compared to fifty-four), and finally because the mean deviation from the mean value is greater.

#### SUMMARY

1. The refractivity of egg albumin is found to increase with heat denaturation in alkaline solution.
2. This increase is interpreted as a structural rearrangement within the protein molecule.

3. The specific refractive increment of egg albumin has been redetermined and found to be  $0.00185 \pm 0.00002$ .

The author wishes to express his appreciation to Professor J. W. McBain for advice and criticism given during the course of this investigation.

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## TRYPTOPHANE METABOLISM

### V. GROWTH ON TRYPTOPHANE-DEFICIENT DIETS SUPPLEMENTED WITH $\beta$ -3-INDOLEACRYLIC, $\alpha$ -OXIMINO- $\beta$ -3-INDOLEPROPIONIC, AND *l*- AND *dl*- $\beta$ -3-INDOLELACTIC ACIDS\*

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The possibility of replacing essential amino acids in the diet of the rat by more or less closely related synthetic compounds has received considerable attention in recent years. Investigations of this nature are important because they indicate the types of chemical reactions which the animal body is capable of effecting.

This communication is a report of such a study of compounds related to tryptophane, specifically  $\beta$ -3-indoleacrylic acid,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid, and the *l* and *dl* forms of  $\beta$ -3-indolelactic acid.

A synthesis of  $\beta$ -3-indoleacrylic acid or of  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid has not previously been recorded in the literature. The conversion of  $\beta$ -3-indoleacrylic acid to tryptophane would require only the directive addition of ammonia to the double-bonded carbons.  $\beta$ -4-Imidazoleacrylic acid has been employed in diets deficient in the corresponding essential amino acid, histidine, by Cox and Rose (1926) and by Harrow and Sherwin (1926). Whereas Cox and Rose found this derivative entirely unable to prevent the loss of weight induced by the lack of histidine, Harrow and Sherwin obtained results indicating some replacement under similar conditions. The possible significance of other  $\alpha$ ,  $\beta$  un-

\* The major portion of this communication was presented before the American Society of Biological Chemists at Cincinnati, April 12, 1933 (*J. Biol. Chem.*, **100**, xii (1933)).

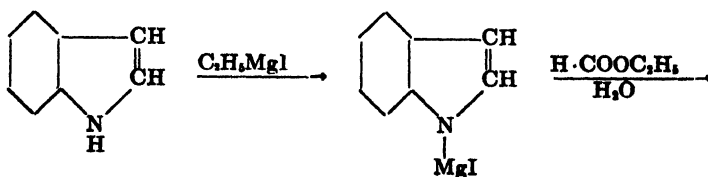
The experimental data in this paper are taken from a dissertation submitted by Lyle C. Bauguess in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

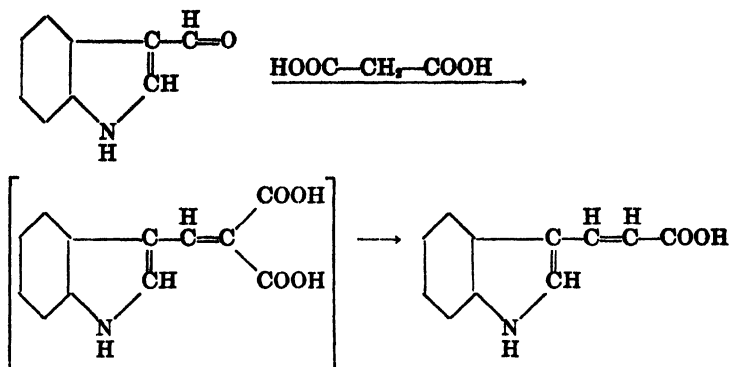
saturated acids as supplements in diets devoid of the corresponding essential amino acid deserves further study. The  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid would replace tryptophane in the diet if it could be either reduced to yield tryptophane itself or hydrolyzed to yield  $\beta$ -3-indolepyruvic acid which is an effective substitute for tryptophane (Jackson, 1929; Berg, Rose, and Marvel, 1929-30). Thus far no similar derivatives have been tested in the animal body. Maurer (1927), however, has shown that actively fermenting yeast can reduce the unsubstituted  $\alpha$ -oximinopropionic acid to the corresponding amino acid, alanine.

Attempts to replace essential amino acids in the diet with the related  $\alpha$ -hydroxy acids have met with varied success. McGinty, Lewis, and Marvel (1924-25) found *dl*- $\alpha$ -hydroxy- $\epsilon$ -aminocaproic acid unable to replace lysine; Cox and Rose (1926) and Harrow and Sherwin (1926) obtained growth on diets containing *dl*- $\beta$ -4-imidazolelactic acid instead of histidine; Westerman and Rose (1928) secured no growth response when a cystine-deficient diet was supplemented with *l*- $\beta$ -dithiodilactic acid. Jackson (1927) and Ichihara and Iwakura (1931) agree that *l*- $\beta$ -3-indolelactic acid cannot be used by the rat as a substitute for tryptophane; however, the results on the product obtained after attempted racemization of this derivative are divergent. Jackson obtained no growth response. Ichihara and Iwakura noted excellent growth. Unpublished data of Heft and Sherwin have been cited by Hawk and Bergeim (1926) as indicating that indolelactic acid cannot replace tryptophane in the diet. No data are given and no mention is made of the optical properties of the compound employed. Because of the probable importance of the  $\alpha$ -hydroxy derivatives in amino acid metabolism, the status of *dl*- $\beta$ -3-indolelactic acid as a dietary substitute for tryptophane deserves further study.

#### EXPERIMENTAL

*$\beta$ -3-Indoleacrylic acid* was synthesized from indole by the steps indicated in the accompanying diagram.  *$\beta$ -3-Indolealdehyde* was

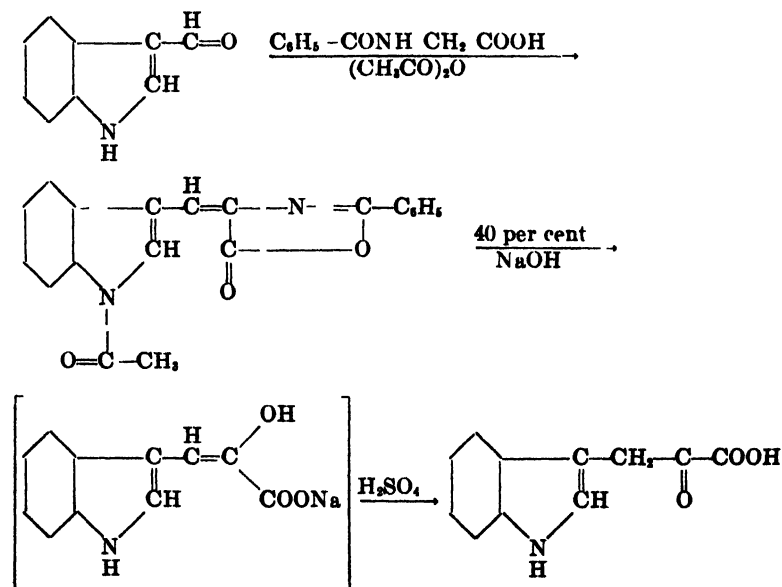


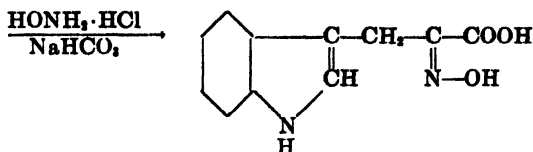


prepared by Putochin's (1926) modification of the method of Majima and Kotake (1922), except that di-*n*-butyl ether was used as a solvent for the preparation and use of the Grignard reagent (Berg, Rose, and Marvel, 1929-30). The  $\beta$ -3-indolealdehyde was condensed with malonic acid essentially according to the general method of Dutt (1924-25) for preparing  $\alpha, \beta$  unsaturated acids. The adaptation of this method found effective was as follows: 1.5 gm. of  $\beta$ -3-indolealdehyde and 3.0 gm. of malonic acid were dissolved in a minimal amount of redistilled pyridine to which were added 3 to 5 drops of piperidine. The solution was allowed to stand at 38-42°, with frequent shaking, for 40 to 60 hours. Higher temperatures caused the formation of resins. The reaction mixture was then poured into 200 cc. of water, acidified with 10 per cent HCl, and set aside in the cold for 6 hours. The flocculent precipitate was filtered off, the filtrate made alkaline to litmus, extracted with ether until pyridine-free, acidified with 10 per cent HCl, and cooled for 6 hours. The precipitate thus obtained was added to the first, and the filtrate discarded. From a total of 15 gm. of  $\beta$ -3-indolealdehyde thus treated, 13.5 gm. of crude  $\beta$ -3-indoleacrylic acid were prepared. This was further treated by dissolving in sodium hydroxide, reprecipitating with acid, repeating the process, and eventually recrystallizing from hot water. 6.5 gm. of glistening reddish brown rhombic platelets were obtained which melted at 195-196° (uncorrected). The neutral equivalent, determined by electrometric titration of an alcohol solution, was found to be 186.3 and the percentage of nitrogen, 7.50. The theoretical values are 187.1 and 7.49, respec-

tively. The compound is soluble in alkalis, ether, alcohol, and hot water; it is relatively insoluble in cold water, dilute acids, and petroleum ether. To establish further the identity of the  $\beta$ -3-indoleacrylic acid, it was reduced in alcohol solution with sodium amalgam, and the  $\beta$ -3-indolepropionic acid resulting was isolated by evaporating the solvent to dryness on a water bath, dissolving the residue in water, acidifying, and clarifying the boiling solution by treating with bone-black and filtering. On cooling the filtrate, characteristic light yellow crystals of indolepropionic acid were obtained. They melted at  $132\text{--}133^\circ$  (uncorrected), contained 7.39 per cent of nitrogen, and gave a neutral equivalent of 187.6. The theoretical values for the per cent of nitrogen and the neutral equivalent are 7.41 and 189.1, respectively. A mixture of this product with that prepared by Berg, Rose, and Marvel (1929-30), having a melting point of  $134^\circ$ , melted at  $133\text{--}134^\circ$  (uncorrected).

$\alpha$ -Oximino- $\beta$ -3-indolepropionic acid was prepared from  $\beta$ -3-indolealdehyde by converting the latter into  $\beta$ -3-indolepyruvic acid according to the procedure of Ellinger and Matsuoka (1920) and condensing this product with hydroxylamine as indicated in the accompanying diagram.



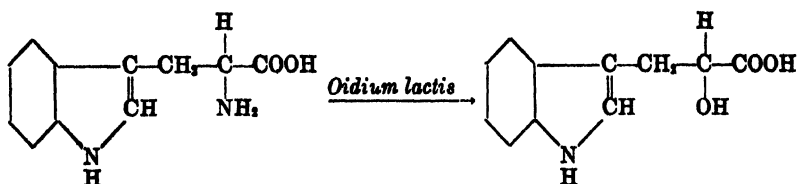


The  $\beta$ -3-indolepyruvic acid prepared darkened at  $200^\circ$ , softened at  $205^\circ$ , and melted at  $211^\circ$  (uncorrected). The *p*-nitrophenylhydrazone melted at  $152$ – $153^\circ$ , in good agreement with the melting point obtained for this derivative by Ellinger and Matsuoka.

7.1 gm. of  $\beta$ -3-indolepyruvic acid were allowed to react in cold sodium bicarbonate solution with an equivalent amount of hydroxylamine hydrochloride, essentially according to the general method of Erlenmeyer (1892) for preparing oximes. The  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid isolated on acidifying, cooling, and filtering the reaction mixture was further purified by dissolving in ether and precipitating with petroleum ether. After two such steps, 7.0 gm. of a tan precipitate were obtained which melted indefinitely somewhat above  $175^\circ$ . The product was soluble in alkalis, ether, alcohol, and hot water, but practically insoluble in petroleum ether and cold water. It contained 12.46 per cent nitrogen and gave a neutral equivalent of 216.5 on electrometric titration. Theoretical values are 12.85 and 218.1, respectively. As further confirmation of the identity of the compound, it was reduced catalytically. The reduction was carried out in alcohol which contained also the Raney catalyst, prepared as directed by Covert and Adkins (1932). The reaction mixture was shaken in the presence of hydrogen at room temperature and pressure somewhat above atmospheric for 12 hours, the catalyst was then filtered off, washed, and the washings and filtrate concentrated to a small volume after decolorization with norit. On cooling for several hours, yellow-tinted crystals of *dl*-tryptophane precipitated. They melted at  $282$ – $284^\circ$ ; a mixture with *dl*-tryptophane (m.p.  $282$ – $283^\circ$ ), prepared by racemizing *l*-tryptophane, melted within the same range. The *dl*-tryptophane prepared by catalytic reduction was found to contain 13.68 per cent of nitrogen; the calculated percentage is 13.72.

*l*- $\beta$ -3-Indolelactic acid was prepared biologically from *l*-tryptophane, by subjecting it to the action of *Oidium lactis* for a period of 4 weeks, according to the method of Ehrlich and Jacobsen (1911).





8.9 gm. of this product were obtained from 22 gm. of *l*-tryptophane. The slightly colored preparation melted at 100–101° (uncorrected). The neutral equivalent was found to be 203.4 and the percentage of nitrogen, 6.80; theoretical values are 205.1 and 6.83, respectively. Ehrlich and Jacobsen report a melting point of 99°; Jackson's (1927) product melted at 100–101° (corrected). The specific rotation of our product was  $[\alpha]_D^{20} = -5.36^\circ$ , which compares favorably with  $[\alpha]_D^{20} = -5.34^\circ$  found by Ehrlich and Jacobsen and  $[\alpha]_D^{25} = -5.37^\circ$  by Jackson.

*dl*- $\beta$ -3-Indolelactic acid was prepared by racemizing the levo isomer. This was accomplished by autoclaving a mixture of 1 part of the derivative (5 gm.) with 3 parts of a 10 per cent aqueous solution of barium hydroxide (anhydrous) at 150–160° for 12 hours. The method is essentially that of Ichihara and Iwakura (1931). The product (4 gm.) isolated from the water solution obtained after the quantitative removal of the barium as barium sulfate melted at 144–145° (uncorrected) and was completely inactive. It contained 6.78 per cent of nitrogen, and possessed a neutral equivalent of 206.7; calculated values are 6.83 and 205.1, respectively. Ichihara and Iwakura record a melting point of 145–146° for their preparation. As a further check on the identity of our product, 0.85 gm. of *dl*- $\beta$ -3-indolelactic acid were prepared by reducing 1 gm. of  $\beta$ -3-indolepyruvic acid in ethyl alcohol solution with hydrogen at low pressure in the presence of the Raney (nickel) catalyst. The product isolated melted also at 144–145° (uncorrected); the melting point of a mixture of the derivatives prepared from the two sources showed no depression.

In conjunction with the preparation of *l*- $\beta$ -3-indolelactic acid a study was made of the possibility of preparing the *dl* derivative from *dl*-tryptophane by the action of *Oidium lactis*. After 4 weeks of incubation of four flasks each of *l*-tryptophane and *dl*-tryptophane containing the same medium (aside from the trypto-

phane isomer used) and inoculated with the same culture of *Oidium lactis*, both the unutilized tryptophane and the indolelactic acid produced were isolated. The mold grew much less profusely on the medium containing the *dl*-tryptophane. The  $\beta$ -3-indolelactic acid isolated was levorotatory in all cases (between  $[\alpha]_D^{20} = -5.2^\circ$  and  $[\alpha]_D^{20} = -5.4^\circ$ ), whether the original medium contained *l*-tryptophane or *dl*-tryptophane. The melting points of the isolated fractions varied between 99–101° (uncorrected). No change from the original value was noted in the specific rotation of the tryptophane isolated from the *l*-tryptophane flasks. In all four cases, however, the tryptophane isolated from the flasks originally containing *dl*-tryptophane showed values for  $[\alpha]_D^{20}$  between +21.5° and +22.0°. In other words, *Oidium lactis* is able to resolve *dl*-tryptophane because of its more ready utilization of the levo isomer. A longer time for incubation would presumably bring the resolution nearer completion. This aspect is being studied further.

Each of the above compounds was tested for its growth-promoting properties by administering it to growing rats as a supplement in a tryptophane-deficient diet. The latter was composed of acid-hydrolyzed casein (*cf.* Berg and Rose, 1929) 14.7, cystine 0.3, starch 39.5, sucrose 15, Crisco 19, cod liver oil 5, salt mixture (Hawk and Oser, 1931) 4.5, and agar 2 per cent, and was fed *ad libitum*. The supplement replaced an equal amount of the acid-hydrolyzed casein. In a few instances separate supplements of the derivative tested were incorporated in the vitamin pills which were fed separately at 12 hour intervals. The latter were composed of 100 mg. of yeast,<sup>1</sup> 50 mg. of starch, and sufficient water to make a stiff dough.

In testing the indoleacrylic acid, two series of animals were employed. In the first series an attempt was made to increase the indoleacrylic acid supplement at the end of 40 days by feeding the derivative in amount equal to 10 mg. of tryptophane separately twice daily, in addition to the supplement in the food mixture (equivalent to 0.2 per cent of tryptophane). When two of the rats thus fed died, the separate supplement was discontinued and the third animal received the derivative from then on only in the food mixture. It survived the 80 days. Due to the deaths result-

<sup>1</sup> The yeast was kindly supplied by the Northwestern Yeast Company, Chicago.

ing in the first series of animals, the experiment was repeated on a second litter of seven rats. Three served as tryptophane controls, receiving 0.2 per cent, 0.1 per cent, and 0.05 per cent of *l*-tryptophane, respectively; one animal received no dietary supple-

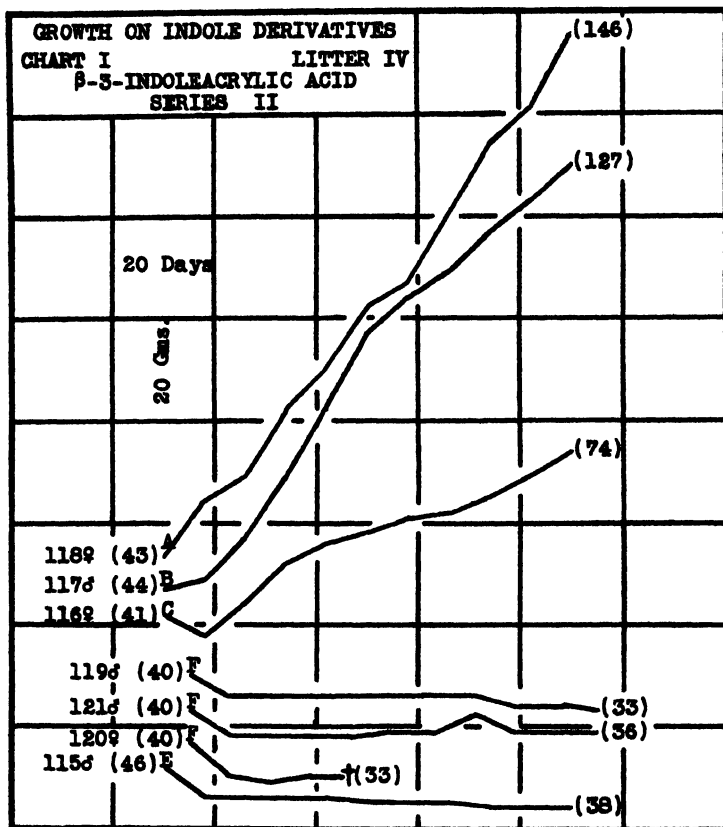


CHART I. Initial and final weights are given in parentheses. Dietary supplement: A represents 0.2 per cent *l*-tryptophane; B, 0.1 per cent *l*-tryptophane; C, 0.05 per cent *l*-tryptophane; F, indoleacrylic acid equivalent to 0.2 per cent tryptophane; E, none. The dagger indicates death.

ment; and three were administered  $\beta$ -3-indoleacrylic acid in amount equivalent to 0.2 per cent of tryptophane, throughout the 80 day period. Only the growth and food consumption data of the second series are recorded in this communication (Chart I

and Table I). The growth curves of the rats receiving the  $\beta$ -3-indoleacrylic acid parallel that of the rat fed the unsupplemented tryptophane-deficient diet, in spite of the fact that the food consumption of those rats was greater.

The  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid was tested on a litter of seven rats, three of which served as controls receiving tryptophane (0.2 per cent, 0.1 per cent, and 0.05 per cent, respectively). Of the others, one served as a no tryptophane control for 40 days; during the second 40 day period it received 10 mg. of tryptophane separately twice daily. Three animals received  $\alpha$ -oximino- $\beta$ -3-

TABLE I  
*Food Consumption and Body Weight Changes on Tryptophane-Deficient Diet  
Supplemented with  $\beta$ -3-Indoleacrylic Acid*

Rat No and sex	Average daily		Supplement
	Change in weight	Food consumption	
	gm	gm	
115 ♂	-0 10	1 2	None
118 ♀	+1 29	7 6	<i>l</i> -Tryptophane
117 ♂	+1 04	5 2	0 2 per cent
116 ♀	+0 41	4 5	0 1 " "
			0 05 " "
			$\beta$ -3-Indoleacrylic acid
119 ♂	-0 09	3 6	0 183 per cent*
120 ♀ †	-0 22	3 3	0 183 " "
121 ♂	-0 05	2 3	0 183 " "

\* Equivalent to 0.2 per cent tryptophane.

† Rat 120 died on the 32nd day. All other animals were fed for 80 days.

indolepropionic acid in amount equivalent to 0.2 per cent of tryptophane throughout the 80 day period. During the second half of the period the derivative was fed also separately twice daily in amount equivalent to 10 mg. of tryptophane. The data (Chart II and Table II) clearly show that  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid cannot replace tryptophane as a growth promoter in the rat. Animals receiving the indole derivative lost weight as rapidly as the control receiving the unsupplemented tryptophane-deficient diet.

In testing the *l*- and *dl*-indolelactic acids, ten rats were employed.

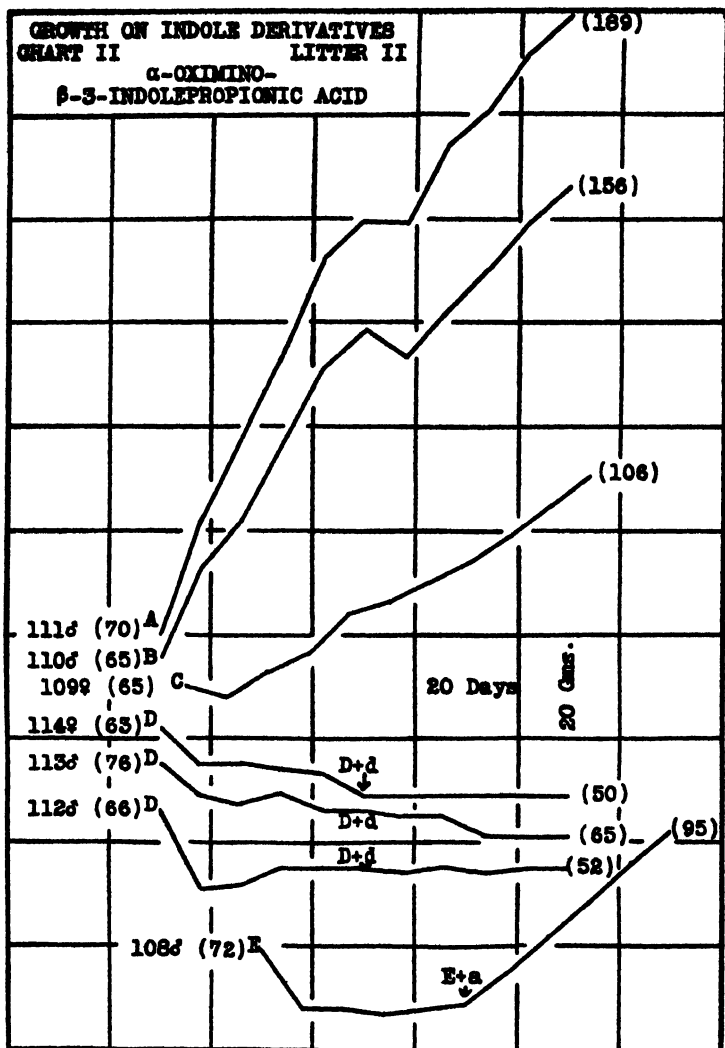


CHART II. Initial and final weights are given in parentheses. Dietary supplement: A represents 0.2 per cent *L*-tryptophane; B, 0.1 per cent *L*-tryptophane; C, 0.05 per cent *L*-tryptophane; D,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid equivalent to 0.2 per cent tryptophane; d,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid equivalent to 10 mg. of tryptophane fed separately twice daily; a, 10 mg. of *L*-tryptophane fed separately twice daily; E, none.

Three pairs served as controls throughout the 80 day experimental period. One pair received 0.2 per cent of *l*-tryptophane in the diet; the second, 0.1 per cent; and the third, none. Two of the four remaining rats received 0.4 per cent of *l*- $\beta$ -3-indolelactic acid, and two 0.2 per cent, for the first and third 20 days of the 80 day experimental period; during the second and fourth 20 days the *l*- $\beta$ -3-indolelactic acid was replaced by an equal amount

TABLE II

*Food Consumption and Body Weight Changes on Tryptophane-Deficient Diet Supplemented with  $\alpha$ -Oximino- $\beta$ -3-Indolepropionic Acid*

Rat No. and sex	Days	Average daily		Supplement
		Change in weight	Food con- sumption	
		gm.	gm.	
108 ♂	1-40	-0.25	2.7	None
	41-80	+0.83	5.2	10 mg. <i>l</i> -tryptophane (fed separately twice daily)
111 ♂	1-40	+1.98	6.5	<i>l</i> -Tryptophane
	41-80	+1.00	7.7	0.2 per cent (in diet)
110 ♂	1-40	+1.58	5.7	0.2 " " " "
	41-80	+0.70	6.5	0.1 " " " "
109 ♀	1-40	+0.40	3.8	0.05 " " " "
	41-80	+0.63	5.1	0.05 " " " "
112 ♂	1-40	-0.23	3.0	$\alpha$ -Oximino- $\beta$ -3-indolepropionic acid
	41-80	-0.13	3.5	0.214 per cent (in diet)*
113 ♂	1-40	-0.28	3.0	Same + 11 mg. separately twice daily
	41-80	0.00	3.7	0.214 per cent (in diet)
114 ♀	1-40	-0.33	3.3	Same + 11 mg. separately twice daily
	41-80	0.00	3.0	0.214 per cent (in diet)

\* Equivalent to 0.2 per cent tryptophane.

of the *dl* modification. Analysis of the data summarized in Chart III and Table III indicates clearly that *l*- $\beta$ -3-indolelactic acid is incapable of supporting growth when fed in conjunction with a diet deficient in tryptophane. Loss of weight was quite as marked in the rats during the 20 days on the levo isomer as it was in the controls receiving no dietary supplement. The results convincingly demonstrate, however, that *dl*- $\beta$ -3-indolelactic acid is cap-

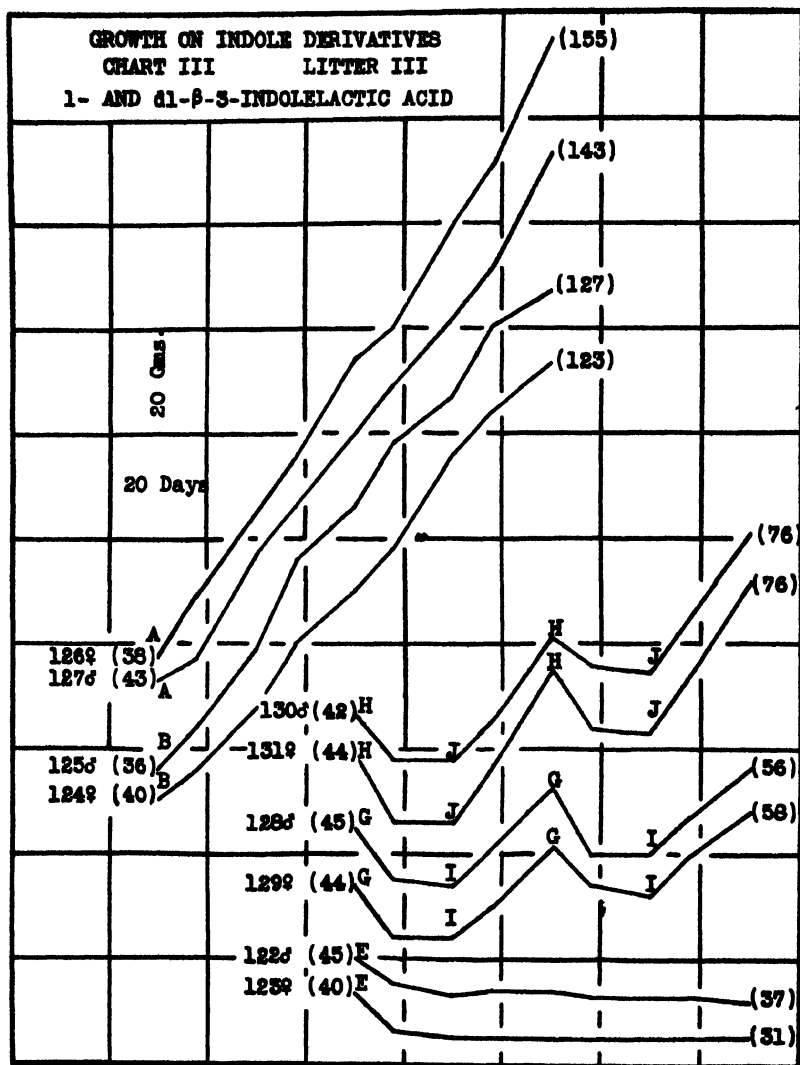


CHART III. Initial and final weights are given in parentheses. Dietary supplement: A represents 0.2 per cent *l*-tryptophane; B, 0.1 per cent *l*-tryptophane; G, *l*-indolelactic acid equivalent to 0.2 per cent tryptophane; H, *l*-indolelactic acid equivalent to 0.4 per cent tryptophane; I, *dl*-indolelactic acid equivalent to 0.2 per cent tryptophane; J, *dl*-indolelactic acid equivalent to 0.4 per cent tryptophane; E, none.

able of serving as a substitute for tryptophane for purposes of growth. This finding is in accord with that of Ichihara and Iwakura (1931) and opposed to that of Jackson (1927). Jackson's conditions for racemization were much less drastic than those employed by Ichihara and Iwakura (1931) which we also adopted.

TABLE III

*Food Consumption and Body Weight Changes on Tryptophane-Deficient Basal Diet Supplemented with l- and dl- $\beta$ -3-Indolelactic Acid*

Days	Rat No. and sex	Average daily		Rat No. and sex	Average daily		Supplement
		Change in weight	Food consumption		Change in weight	Food consumption	
		gm.	gm.		gm.	gm.	
1-20	122 ♂	-0.35	3.5	123 ♀	-0.45	3.1	None
21-40		+0.05	3.4		0.00	3.5	"
41-60		-0.05	2.8		0.00	3.4	"
61-80		-0.05	2.9		0.00	3.0	"
							Indolelactic acid
1-20	128 ♂	-0.55	3.2	129 ♀	-0.50	3.3	0.2 per cent (l-)
21-40		+0.90	7.0		+0.85	7.8	0.2 " " (dl-)
41-60		-0.60	3.4		-0.45	3.5	0.2 " " (l-)
61-80		+0.80	7.7		+0.80	8.0	0.2 " " (dl-)
1-20	130 ♂	-0.45	2.7	131 ♀	-0.65	3.1	0.4 " " (l-)
21-40		+1.15	8.1		+1.45	7.9	0.4 " " (dl-)
41-60		-0.30	3.5		-0.60	3.3	0.4 " " (l-)
61-80		+1.30	8.0		+1.40	7.9	0.4 " " (dl-)
							l-Tryptophane
1-20	125 ♂	+1.15	6.5	124 ♀	+0.90	7.0	0.1 per cent
21-40		+1.35	7.2		+1.10	6.5	0.1 " "
41-60		+1.05	6.1		+1.30	6.5	0.1 " "
61-80		+1.00	7.7		+0.85	7.4	0.1 " "
1-20	127 ♂	+1.20	7.8	126 ♀	+1.35	7.9	0.2 " "
21-40		+1.10	7.9		+1.45	8.0	0.2 " "
41-60		+1.15	7.5		+1.30	7.8	0.2 " "
61-80		+1.55	7.3		+1.75	7.5	0.2 " "

He refluxed his preparation of l- $\beta$ -3-indolelactic acid in 100 cc. of water, to which had been added also 10 gm. of barium hydroxide (the hydrate), for 10 hours at a temperature of about 100°. In the method of Ichihara and Iwakura, the 10 per cent barium hydroxide solution was based on the anhydrous form and the tem-



perature was maintained between 150–160° for 7 or more hours. Furthermore, Jackson states, "The melting point was 98–100°C. (corrected). There was no doubt that the recovered substance was indole lactic acid. Owing both to the small amount of material and lack of facilities at the time, measurements of the optical activity were not undertaken." The preparation recorded in this communication, made by the method of Ichihara and Iwakura, was completely inactive. It melted at 144–145° (uncorrected), in agreement with that for *dl*- $\beta$ -3-indolelactic acid prepared by reducing  $\beta$ -3-indolepyruvic acid (and with that obtained by Ichihara and Iwakura, as previously stated), but considerably above the 100–101° melting point of *l*- $\beta$ -3-indolelactic acid. There is little doubt, therefore, that the conditions employed by Jackson were not sufficiently drastic to racemize his preparation. Although the average daily growth rates of our rats receiving 0.4 per cent of the *dl*- $\beta$ -3-indolelactic acid (+1.33) compare well with those of the control rats on 0.2 per cent of tryptophane (+1.36), the correlation was not as favorable between the findings on animals receiving the 0.2 per cent of *dl*- $\beta$ -3-indolelactic acid (+0.84) and those on the animals receiving 0.1 per cent of tryptophane (+1.09). In any event, the response indicates that the dextro component of the *dl*- $\beta$ -3-indolelactic acid possesses considerable efficiency in replacing tryptophane. The slight difference in response on equivalent supplements of the effective optical isomer and tryptophane may indicate that the conversion of the former into the latter, particularly when fed at suboptimum levels, is either not sufficiently complete or not sufficiently rapid to permit the indole derivative to meet as fully the demands of the growing animal for the amino acid. The observations on *dl*- $\beta$ -3-indolelactic acid, as compared with those on *l*- $\beta$ -3-indolelactic acid, emphasize the importance of considering optical activity in animal experiments of this type. The above findings require the classification of tryptophane with histidine (Cox and Rose, 1926; Harrow and Sherwin, 1926) as being replaceable by the corresponding  $\alpha$ -hydroxy acids, rather than with lysine (McGinty, Lewis, and Marvel, 1924–25) or cystine (Westerman and Rose, 1928) which are not thus replaceable.

## SUMMARY

$\beta$ -3-Indoleacrylic acid,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid, and *l*- and *dl*- $\beta$ -3-indolelactic acid have been prepared and tested for their ability to replace the essential amino acid, tryptophane, in the diet of the rat. Of the compounds, none except *dl*- $\beta$ -3-indolelactic acid was effective.

*dl*- $\beta$ -3-Indolelactic acid, when fed as a supplement in a tryptophane-deficient diet, induced growth at a rate which indicated that the dextro component possessed considerable efficiency in replacing tryptophane.

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## TRYPTOPHANE METABOLISM

### VI. THE PRODUCTION OF KYNURENIC ACID FROM INDOLE DERIVATIVES\*

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The fact that a compound can replace tryptophane in the diet of the rat cannot be used with any degree of certainty as a basis for predicting its utilization in the rabbit for kynurenic acid production. The reverse is also true. Whereas, for example, tryptophane ethyl ester hydrochloride and acetyltryptophane both promote good growth in the rat when used as supplements in a tryptophane-deficient diet (Berg, Rose, and Marvel, 1929-30, *a*), only the former produces kynurenic acid in the rabbit as readily as does free tryptophane (Berg, 1931); the yield from acetyltryptophane is considerably smaller. *d*-Tryptophane promotes as good growth as *l*-tryptophane (du Vigneaud, Sealock, and Van Etten, 1932; Berg, 1934), but produces so small an output of kynurenic acid as to indicate little or no conversion into that substance (Berg, 1934). Kynurenine, on the other hand, is unable to replace tryptophane in the diet of the rat (Jackson and Jackson, 1932), but does effectively produce kynurenic acid in the rabbit (Kotake and Iwao, 1931).

Hence, having employed  $\beta$ -3-indoleacrylic acid,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid, and *l*- and *dl*- $\beta$ -3-indolelactic acid in growth promotion studies (Bauguess and Berg, 1934), we were interested in studying these derivatives, and  $\beta$ -3-indolepyruvic acid, also from the standpoint of kynurenic acid production.  $\beta$ -3-Indolepyruvic acid has been tested for its growth-promoting properties

\* The experimental data in this communication are taken from a dissertation submitted by Lyle C. Bauguess in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

by several investigators. Jackson (1929) and Berg, Rose, and Marvel (1929-30, b) secured excellent growth in rats fed tryptophane-deficient diets supplemented with this derivative. Contrary results have been obtained by Heft and Sherwin (unpublished data cited in Hawk and Bergeim, 1926) and by Ichihara and Iwakura (1931). The latter apparently based their evidence on the weight loss of one rat during a 4 day test period. The balance of evidence strongly favors the view that  $\beta$ -3-indolepyruvic acid is utilized well for growth. On the other hand, Ellinger and Matsuoka (1920) found a relatively much smaller conversion of this derivative, than of *l*-tryptophane, into kynurenic acid. Of the remaining compounds, only *dl*-indolelactic acid is an effective substitute for tryptophane as a growth promoter (Ichihara and Iwakura, 1931; Bauguess and Berg, 1934). Ichihara and Iwakura have tested *dl*-indolelactic acid also for its conversion into kynurenic acid. Using two rabbits, they obtained a small yield in one and none in the second. The possible importance of indolepyruvic acid and indolelactic acid as intermediates in the production of kynurenic acid from tryptophane, as well as in the oxidation of that amino acid, justifies further study to supplement the limited data available on these derivatives. *l*- $\beta$ -3-Indolelactic acid,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid, and  $\beta$ -3-indoleacrylic acid have not previously been employed in kynurenic acid production experiments.

#### EXPERIMENTAL

The synthesis of each of the compounds used in these studies has been outlined in the previous communication (Bauguess and Berg, 1934). Each was checked for purity by determining its melting point, mixed melting point (with previous preparation), nitrogen content (by Kjeldahl), and molecular weight (by titration). The optical purity of the *l*- and *dl*- $\beta$ -3-indolelactic acids was also established. In each instance, the value obtained accorded well with that found in the preceding paper. The *l*-tryptophane employed was prepared according to the method of Cox and King (1930). The nitrogen found was 13.65, as compared with the theoretical 13.72 per cent. The  $[\alpha]_D^{20}$ , determined on a 0.5 per cent solution in water, was  $-33.0^\circ$ . This agrees well with  $[\alpha]_D$  values recorded in the literature for *l*-tryptophane polarized under similar conditions (*cf.* Berg and Potgieter, 1931-32).

The technique employed in testing the above compounds for kynurenic acid production has been discussed elsewhere (Berg, 1931) in detail. Essentially it consists in the administration of the substance to male rabbits (either subcutaneously or orally), the collection of the 24 hour urine following, and the isolation of the kynurenic acid by the Capaldi (1897) procedure. In these, as in previous experiments, the kynurenic acid precipitates were washed routinely with water-saturated butyl alcohol which was found effective in removing such of the indole derivatives or of their metabolites as were excreted and coprecipitated. The animals were kept in individual metabolism cages and given water and

TABLE I  
*Kynurenic Acid Recoveries by Capaldi Procedure*

Rabbit No.	Weight of partially purified kynurenic acid used	Net weight of kynurenic acid isolated after washing with 5 cc. butyl alcohol	Recovery
	gm.	gm.	per cent
1	0.1500 added	0.1305	87.0
2	0.1500 "	0.1292	86.1
3	0.1500 "	0.1348	89.9
4	0.1500 "	0.1335	89.0
1	0.1500 injected subcutaneously	0.1282	85.5
2	0.1500 " "	0.1296	86.4
3	0.300 " "	0.2852	95.1
4	0.300 " "	0.2649	88.3

oats *ad libitum*. The compounds were administered every 3rd day. All 24 hour urines, whether collected for control or experimental periods, were analyzed according to the routine procedure. In control periods a scanty amorphous precipitate was always obtained.

As a check on the general procedure, the sodium salt of partially purified kynurenic acid (*cf.* Berg, 1931) was either (a) added to some of the urine of the animals employed, or (b) injected subcutaneously, and its net recovery determined in each case as the difference between the amount of precipitate obtained from these urines and that obtained from comparable urines (a) to which no crude kynurenic acid was added, or (b) collected during similar

periods when kynurenic acid was not injected. In all cases, the precipitates were washed with 5 cc. of water-saturated *n*-butyl alcohol. The results of these tests appear in Table I. They show that the procedure is fairly quantitative. The recoveries after subcutaneous injection serve to substantiate the findings of Kotake and Ichihara (1931) that kynurenic acid does not undergo appreciable oxidation in the rabbit or appreciable excretion in the

TABLE II  
*Kynurenic Acid Elimination Following Subcutaneous Administration of Indole Derivatives*

Day	Kynurenic acid precipitate after washing with 5 cc butyl alcohol		Substance administered (2 equal doses, 9 hrs. apart, as sodium salt)
	Rabbit 1 ♂, 2.7 kilos	Rabbit 2 ♂, 3.1 kilos	
	gm.	gm.	
1-2	0.0009	0.0009	1 gm. <i>l</i> -tryptophane
3	0.1933	0.1821	
4-5	0.0009	0.0015	
6	0.0740	0.0434	0.995 gm. $\beta$ -3-indolepyruvic acid
7-8	0.0019	0.0018	1.068 gm. $\alpha$ -oximino- $\beta$ -3-indolepropionic acid
9	0.0021*	0.0027	
10-11	0.0010	0.0015	
12	0.0009	0.0026	1.005 gm. <i>l</i> - $\beta$ -3-indolelactic acid
13-14	0.0011	0.0016	1.005 gm. <i>dl</i> - $\beta$ -3-indolelactic acid
15	0.0201	0.0181	
16-17	0.0019	0.0025	
18	0.0024*	0.0020*	0.916 gm. $\beta$ -3-indoleacrylic acid
19-20	0.0033	0.0030	

The values for days 1 to 2, etc., are the averages per day.

\* In all cases thus indicated, an extra 5 cc. of butyl alcohol were required to remove the coprecipitated indole compounds.

bile. The butyl alcohol washings, as employed, extract 3 to 4 mg. of kynurenic acid, which, if added in each case, would slightly increase the percentage recovered.

The synthetic indole derivatives, and the *l*-tryptophane, were administered both subcutaneously and by mouth in amounts molecularly equivalent to 1 gm. of tryptophane. The kynurenic acid production data are recorded in Tables II and III. It will be noted that, of the derivatives tested, only two,  $\beta$ -3-indolepy-

ruvic acid and *dl*- $\beta$ -3-indolelactic acid, led to an appreciable excretion of kynurenic acid. Following the administration of  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid, precipitates ranging from 0.0611 to 0.4361 gm. were obtained before washing with the butyl alcohol, in which reagent the bulk of each precipitate was readily soluble. The residue resulting was no greater in amount than the precipitate obtained in control periods. Not over 7 or 8 mg. of kynurenic

TABLE III

*Kynurenic Acid Elimination Following Administration of Indole Derivatives by Stomach Tube*

Day	Kynurenic acid precipitate after washing with 5 cc. butyl alcohol		Substance administered (2 equal doses, 9 hrs. apart, as sodium salt)
	Rabbit 3 $\sigma$ , 2.8 kilos	Rabbit 4 $\sigma$ , 3.2 kilos	
	gm.	gm.	
1-2	0.0004	0.0004	
3	0.2507	0.2042	1 gm. <i>l</i> -tryptophane
4-5	0.0010	0.0009	
6	0.0655	0.0596	0.995 gm. $\beta$ -3-indolepyruvic acid
7-8	0.0021	0.0009	
9	0.0010*	0.0011*	1.068 gm. $\alpha$ -oximino- $\beta$ -3-indolepropionic acid
10-11	0.0014	0.0015	
12	0.0015	0.0019	1.005 gm. <i>l</i> - $\beta$ -3-indolelactic acid
13-14	0.0019	0.0020	
15	0.0211	0.0289	1.005 gm. <i>dl</i> - $\beta$ -3-indolelactic acid
16-17	0.0021	0.0022	
18	0.0019*	0.0019*	0.916 gm. $\beta$ -3-indoleacrylic acid
19-20	0.0017	0.0017	

The values for days 1 to 2, etc., are the averages per day.

\* In all cases thus indicated, an extra 5 cc. of butyl alcohol were required to remove the coprecipitated indole compounds.

acid could have been removed in the washing process, had that amount been present. In each instance, the substance isolated from the butyl alcohol washings gave a strongly positive Hopkins-Cole test and was, therefore, checked by melting point and mixed melting point determinations with a sample of the original derivative.  $\alpha$ -Oximino- $\beta$ -3-indolepropionic acid melts somewhere above 175° (uncorrected). The precipitates melted between 165-175° and the mixed melting points were all intermediate. Undoubtedly



the greater proportion of the material washed out by the butyl alcohol was the unaltered derivative. The  $\beta$ -3-indoleacrylic acid, likewise, was found to yield large quantities (0.6407 to 0.7572 gm.) of butyl alcohol-soluble material responding strongly to the Hopkins-Cole test.  $\beta$ -3-Indoleacrylic acid melts at 195–196° (uncorrected). The melting points of the substances isolated from the butyl alcohol washings varied between 186–190° and 193–196° (uncorrected). Mixed melting points with samples of the original derivative were intermediate, in each instance. There is little doubt that  $\beta$ -3-indoleacrylic acid is largely excreted, either as such or as a conjugated product which yields the derivative when the urine is subjected to the Capaldi procedure for isolating kynurenic acid. It is important to note that by more vigorous procedures still greater amounts of this and of the previous derivative would likely have been recovered.

Following the administrations of *l*-tryptophane,  $\beta$ -3-indolepyruvic acid, and *l*- and *dl*- $\beta$ -3-indolelactic acid, no appreciable coprecipitation with kynurenic acid occurred. In fact, the quantity of material removed by the butyl alcohol washings did not, in any instance, exceed that removed from the precipitates for the control period. On applying the Hopkins-Cole test to the urine following the *per os* administrations of the above compounds, a positive response was obtained in every case; urine following the administration of indolelactic acid was particularly strongly positive. After subcutaneous administration of indolelactic acid, the urine gave an intense color with the reagents; after indolepyruvic acid, a very doubtful test; and after *l*-tryptophane, none at all. In previous studies we have found that all 24 hour urines, following *per os* administration of 1 gm. of *l*-tryptophane or less, give positive tests with the Hopkins-Cole reagent, whereas they do not thus respond after the subcutaneous injection of the amino acid. The discrepancy is likely due to the presence in the urine of putrefactive products, or their conjugates, which originate in the intestinal tract as a result of bacterial decomposition, and are subsequently absorbed, detoxified, and excreted with the indole ring intact. At any rate, the Hopkins-Cole test is of value when applied to urine as a means of determining qualitatively the completeness of utilization or oxidation of indole derivatives only when such derivatives have been administered by methods other

than *per os*. Since the Hopkins-Cole tests on urine after administration of indolepyruvic acid were of uncertain significance, the isolation of any indole derivative which may have been present was attempted by extracting the acidified urine (from which the kynurenic acid had been precipitated) with butyl alcohol (in which indolepyruvic acid is soluble) and evaporating the solvent under diminished pressure. A positive Hopkins-Cole test was secured on all four extracts, but no appreciable residue was obtained. It seems probable, therefore, that the oxidation or utilization of the  $\beta$ -3-indolepyruvic acid was fairly complete. The isolation of the *l*- and *dl*- $\beta$ -3-indolelactic acids from the urine following their administration was more successful. The procedure consisted essentially in extracting the urine (from which the kynurenic acid had been removed) with ether and precipitating the indolelactic acid from the ether extract with ligroin, as directed by Ehrlich and Jacobsen (1911) for its original preparation. The product isolated was reprecipitated by dissolving it in ether and again adding ligroin. After the *l*- $\beta$ -3-indolelactic acid (m.p. 99–101°, uncorrected) administrations, from 0.0382 to 0.3001 gm. of substance melting from 94–96° to 99–100° (uncorrected) was isolated by the above procedure. Melting points of mixtures of the original product with the fractions isolated from the urine ranged between 96–98° and 99–100° (uncorrected). Obviously the material isolated was the original *l*- $\beta$ -3-indolelactic acid. Following the administration of *dl*- $\beta$ -3-indolelactic acid (m.p. 144–145°, uncorrected), fractions varying from 0.1009 to 0.3842 gm. were isolated. All of these melted between 139–142° and 142–143° (uncorrected) and showed mixed melting points with samples of the original derivative between 142–143° and 144–145° (uncorrected). In three instances, further precipitates, varying in amount from 0.0231 to 0.0334 gm., and melting from 96–97° to 99–100° were obtained from the ether-ligroin filtrate. Mixtures with *l*- $\beta$ -3-indolelactic acid (m.p. 99–101°, uncorrected) gave melting point values intermediate in each case. No such second precipitate was obtained from the ether extract of the fourth urine. The dextro component of the *dl*- $\beta$ -3-indolelactic acid was obviously the more readily utilized in the body, whether for kynurenic acid production, oxidation, or otherwise. Hence, a partial resolution of the *dl* modification resulted. The *dl* mixture was more readily

isolated from the extract than the *l* component, presumably because of its lower solubility. The observations are in essential agreement with those of Ichihara and Iwakura (1931). The greater recovery of *d*- $\beta$ -3-indolelactic acid (half of the *dl* modification) than of  $\beta$ -3-indolepyruvic acid in these studies, though not conclusive evidence, is at least favorable to the view that the latter is the more likely intermediate in the oxidation of tryptophane in the animal organism.

A study of Tables II and III shows that the average amount of kynurenic acid produced from  $\beta$ -3-indolepyruvic acid (0.0606 gm.) and from *dl*- $\beta$ -3-indolelactic acid (0.0241 gm.) was decidedly smaller than that from an equivalent amount of *l*-tryptophane (0.2076 gm.). Melting points of the former in every instance compared well with the melting points of the kynurenic acid obtained after *l*-tryptophane administration and no depression occurred on mixing with the latter before melting. The identity of the precipitates as kynurenic acid was established also by applying the Jaffe (1882-83) and Kretschy (1881) color tests. We are inclined to interpret the wide divergence between the amount of kynurenic acid excreted after the *l*-tryptophane administration and after the administration of either of the two non-amino acids as indicating that neither *d*- $\beta$ -3-indolelactic acid nor  $\beta$ -3-indolepyruvic acid is a normal intermediate in the production of kynurenic acid from *l*-tryptophane. Otherwise one might reasonably expect more favorable comparisons in the amounts of kynurenic acid produced (allowing, of course, for the fact that only half of the *dl*- $\beta$ -3-indolelactic acid is convertible to kynurenic acid).

The data obtained suggest that the mechanisms for the oxidation of *l*-tryptophane and for the production of kynurenic acid from that amino acid are independent of each other. In the former process, indolepyruvic acid may be a normal metabolite; in the latter it probably has no rôle. Its partial conversion (or that of *d*-indolelactic acid) into kynurenic acid may be explained by its previous partial conversion into *l*-tryptophane which then undergoes metabolism by a different path to produce kynurenic acid. Of the several routes suggested, only that of Kotake (1931) has been supported by the isolation of an intermediate (Kotake and Iwao, 1931) which can undergo conversion into kynurenic acid in the animal as readily as can *l*-tryptophane.

## SUMMARY

Several indole derivatives closely related structurally to tryptophane have been tested for their conversion to kynurenic acid in the rabbit. Of these,  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid,  $\beta$ -3-indoleacrylic acid, and *l*- $\beta$ -3-indolelactic acid yielded no appreciable quantity. *dl*- $\beta$ -3-Indolelactic acid and  $\beta$ -3-indolepyruvic acid, on the other hand, were converted into kynurenic acid, but much less completely than was *l*-tryptophane.

After the administration of  $\alpha$ -oximino- $\beta$ -3-indolepropionic acid,  $\beta$ -3-indoleacrylic acid, and *l*- $\beta$ -3-indolelactic acid, each was partially recovered from the urine. Following the administration of *dl*- $\beta$ -3-indolelactic acid, fractions identified as *dl*- and as *l*-indolelactic acid were obtained. No appreciable amount of  $\beta$ -3-indolepyruvic acid was recovered.

The possible metabolic significance of *d*- $\beta$ -3-indolelactic acid and of  $\beta$ -3-indolepyruvic acid, as suggested in part by the data obtained, has been discussed.

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# THE CORTICAL HORMONE REQUIREMENT OF THE ADRENALECTOMIZED DOG, WITH SPECIAL REFERENCE TO A METHOD OF ASSAY\*

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A method of assay for the adrenal cortical hormone utilizing the blood urea level of the adrenalectomized dog was suggested by Harrop, Pfiffner, Weinstein, and Swingle (1, 2) as a result of earlier cooperative work on the effect of the hormone in the normal and adrenalectomized animal. In those studies it was found that the cortical hormone had no demonstrable effect in the normal dog. A rise in blood urea was observed to be one of the first manifestations of insufficiency in the adrenalectomized dog on the gradual withdrawal of extract. A dog unit was defined as "the minimum daily kilogram dose of cortical hormone necessary to maintain normal physiological conditions in the adrenalectomized dog for a period of 7 to 10 days; the two criteria of normal physiological condition being maintenance of body weight and blood level of non-protein nitrogen (or urea)." Rogoff and Stewart (3) had found in their studies on the effect of adrenalectomy in the dog that a significant rise in the blood urea usually terminated the "period of good health" and occurred some time (usually at least several days) before death.<sup>1</sup> All other methods of assay so far

\* In the collaborative studies on the adrenal cortex carried on in this laboratory the writers have specialized in certain phases of the experiments. The physiological aspect of the problem is the responsibility of the senior author (W. W. S.); the biochemical work of extraction and purification of the hormone, that of one of us (J. J. P.). An abstract of this paper was read before the American Society of Biological Chemists at Cincinnati, April 10-12, 1933.

These investigations have been aided by a grant from the Josiah Macy, Jr. Foundation.

<sup>1</sup> Marshall and Davis (4) were the first to observe an increase in the blood urea following adrenalectomy. These observations were made on the cat.

suggested have employed either the rat or cat. A discussion of these techniques and the factors affecting the results are contained in the literature cited (5-15).

Knowledge of the influence of various factors, both physiological and environmental, on the hormone requirement of the individual animal, is necessary for extraction and fractionation studies. This paper is concerned with the quantitative effect of these factors.

*Adrenal Extracts Employed*—The experimental work (with a few exceptions noted in the text) was carried out with a single lot of extract (Extract 31632) prepared by methods previously described (16) from 40 kilos of whole beef adrenals which were received in the laboratory between February 17 and March 16, 1932. The adrenalin concentration<sup>2</sup> of this particular extract was 1:1,000,000. In those instances in which an extract other than this lot was employed the number of the extract is specified. Unassayed stock extract was used to maintain the animals from operation to experimentation, in realimentation, and maintenance between experimental runs. These extracts were prepared so that 1 cc. represented 40 gm. of whole gland.<sup>3</sup> *All dosages of extract are expressed in terms of gm. of whole adrenal gland per kilo of body weight per day unless otherwise stated.*

*Care and Maintenance of Adrenalectomized Dog*—The present study was conducted on a series of ten adrenalectomized adult mongrel dogs, nine males and one female.<sup>4</sup> The adrenal glands

<sup>2</sup> We wish to thank Mr. L. W. Rowe of the Research Laboratories of Parke, Davis and Company for the adrenalin bioassays conducted on the extracts employed in this study.

<sup>3</sup> Unassayed stock extract was prepared by the usual methods (16). The yield of cortical hormone as indicated by bioassay of representative batches was 2000 dog units or more per kilo of whole beef adrenal. Since September, 1932, stock extract was prepared by the so called "half method" (17). Assays conducted on samples drawn from various lots prepared by this method during a period of 12 months production showed the yield to run consistently between 1000 and 2000 dog units per kilo of gland. Methods of extraction will be discussed more fully elsewhere.

<sup>4</sup> At the present writing (October 23, 1933) six of the animals (Dogs 2, 7, 12, 23, 42, and 92) are alive and in good condition, 853, 785, 755, 652, 524, and 272 days, respectively, since adrenalectomy. The other four dogs died 119 to 329 days following the removal of the second adrenal. Autopsy in these four cases failed to reveal any accessory tissue. These data demonstrate

were removed in two stages, first the right and then the left gland. For several days following the removal of the left gland the animals received a dose of 20 to 40 gm. of extract, gradually dropping to a level of 4 to 8 gm. This was the usual dose for maintaining animals between assays. In bringing animals out of an insufficiency period a dose of 10 to 40 gm. was employed, depending upon the severity of the condition. Extract was administered subcutaneously in divided doses unless otherwise stated. Animals in very severe insufficiency were treated intravenously. In the present study a 7 day period was employed for each dosage level.

The animals were housed in individual cages in heated quarters. They received no exercise other than that which they could obtain in their cages. They were fed once a day usually between 10 and 12 a.m. The maintenance diet consisted of prepared dog foods, Diet B,<sup>5</sup> and Diet K,<sup>6</sup> or a mixture of both to which the following supplements were added three times weekly, 10 gm. of yeast,<sup>7</sup> 5 cc. of cod liver oil, 5 gm. of Cowgill's salt mixture (18), and 0.4 gm. of iron and ammonium citrate. The animals were allowed approximately 80 calories of food per kilo of body weight per day.<sup>8</sup>

that adrenalectomized dogs can be maintained for periods of 2 years or longer in apparently normal physiological condition even though they have been allowed to pass repeatedly into insufficiency by withdrawal of extract. It perhaps should be pointed out, however, that two abnormalities have been recognized in this colony of adrenalectomized dogs. In all cases an anemia of varying degree of severity has been observed to develop within the first few months following adrenalectomy. Unoperated control dogs have maintained a normal hemoglobin level upon the same dietary regimen. The quantity of blood taken from these assay animals is negligible when compared to the amount found necessary by Whipple and others to produce hemorrhagic anemia. Parasitic infection has been ruled out as a possible source of hemoglobin loss. Thus far various forms of therapy have failed to correct the condition.

The other abnormality observed in adrenalectomized dogs maintained in good physical condition with ample doses of cortical hormone is an abnormal glucose tolerance curve. The foregoing points will be discussed in detail elsewhere.

<sup>5</sup> Bal Ra, Valentine Meat Juice Company, Richmond, Virginia.

<sup>6</sup> Ken-L-Ration, Chappel Brothers, Rockford, Illinois.

<sup>7</sup> Northwestern Yeast Company, Chicago.

<sup>8</sup> In connection with the high concentration of vitamin C in the adrenal cortex as demonstrated by Szent-Györgyi and others, it is of interest to note here that the diet employed in these experiments contains very little



A daily record of food consumption was kept for each animal. Those which lost weight were allowed to recover their weight losses between assays. Blood samples were obtained at least 15 to 18 hours after all food had been withdrawn.

The method of Folin and Svedberg (19) was used in determining the blood urea nitrogen with the exception of some of the more recent figures which were obtained with the gasometric hypobromite method of Van Slyke (20). Hemoglobin was estimated by Newcomer's method. Blood sugar was determined on a tungstic acid filtrate with the Somogyi (21) modification of the Shaffer-Hartmann reagent.

*Hormone Requirement As Influenced by Physiological Factors—*

An assay on a series of nine adrenalectomized dogs, eight males and one female in anestrus, shows what variation may be expected in the hormone requirement of individual animals. A summary of the data appears in Table I, Series A. Occasionally an animal brought into insufficiency by gradual reduction of dosage level will show a marked fluctuation in the level of blood urea while exhibiting no clinical symptoms of insufficiency other than a somewhat variable appetite. A comparison was made, therefore, of assay results based upon a 100 per cent urea rise and upon clinical failure. The minimum maintenance dose<sup>9</sup> determined by the urea rise in the eight dogs in which this was determined ranged from 0.2 to 0.5 gm., with an average of 0.38 gm. On the basis of clinical failure which includes anorexia, asthenia, and usually vomiting, the minimum maintenance dose for 9 days ranged from 0.2 to 0.4 gm., average 0.29 gm. The same dog showed a variation in minimum maintenance dose by these two criteria of 0 to 108 per cent. There is no correlation between minimum maintenance dose and

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if any vitamin C as demonstrated in feeding experiments with guinea pigs. Several assay animals have been maintained on this dietary régime for periods of 2 years and longer without their adrenal glands but with adrenal extracts containing no demonstrable quantity of vitamin C. Large quantities of vitamin C (decitrated and concentrated lemon juice) orally administered had no effect on the cortical hormone requirement of adrenalectomized dogs. Experimental details will be published elsewhere.

<sup>9</sup> The minimum maintenance dose is the dose the animal was receiving during the period prior to the 100 per cent rise in urea nitrogen or prior to clinical failure as the case may be.

the time interval elapsing between operation and assay. The results are also independent of the number of previous assays. The average weight loss during an assay was 10 per cent. However, dogs can exhibit marked insufficiency and die at a peak weight.

TABLE I

*Cortical Hormone Requirement of a Series of Nine Adrenalectomized Dogs\**

Dog No.	Series	1st day of assay	Days from second operation to assay	Previous insufficiencies	Maximum† and final body weight during assay	Body weight at time of 100 per cent urea rise	Minimum maintenance dose		Falling dose		Diet; plus supplements
							Based on 100 per cent urea rise	Based on clinical symptoms	Based on 100 per cent urea rise	Based on elapsed symptoms	
					kg.	kg.	gm. gland	gm. gland	gm. gland	gm. gland	
92	A	Feb. 21, 1933	27	0	10.9-9.5	10.2	0.34	0.34			K
42	"	July 20, 1932	62	1	8.1-7.3	§		0.4			"
25	"	May 13, 1932	98	3	14.0-11.6	14.0	0.4	0.28	0.34	0.24	"
	B	July 21, 1932		4	14.1-14.1	13.5			0.24	0.20	"
23	A	May 3, 1932	113	4	9.5-8.8	9.0	0.5	0.24	0.40	0.22	"
	B	July 21, 1932		5	9.4-9.4	9.4			0.16	0.16	"
20†	A	Apr. 20, 1932	118	3	9.5-9.1	9.1	0.5	0.28			"
12	"	" 20, 1932	204	5	10.2-9.6	9.8	0.4	0.22	0.34	0.20	"
	B	July 22, 1932		6	10.2-9.0	9.0			0.40	0.40	BH
10	A	May 3, 1932	224	5	15.0-15.0	15.0	0.2	0.2			K
7	"	" 3, 1932	234	6	10.0-8.4	9.0	0.34	0.34	0.28	0.28	"
	B	July 31, 1932		7	10.0-8.2	8.6			0.40	0.34	B
2	A	May 2, 1932	314	5	13.2-11.2	12.2	0.4	0.28	0.34	0.24	K
	B	July 21, 1932		6	12.7-11.0	11.9			0.34	0.28	B

\* The following dosage levels were used: in Series A, 4.0, 2.0, 1.0, 0.5, 0.4, 0.34, 0.28, 0.24, 0.22, 0.2 gm. of gland per kilo per day; in Series B, 4.0, 0.4, 0.34, 0.28, 0.24, 0.22, 0.2 gm.

† The maximum weight is recorded rather than the initial weight since several of the animals gained weight during the early part of the assay.

‡ Diet K, Ken-L-Ration; Diet B, Bal Ra; Diet BH, beef heart.

§ Not determined.

|| A 10 to 29 day period elapsed between check assays.

¶ Female in anestrus; all other dogs males.

The duration of the assay periods, in Table I, ranged from 20 to 70 days. This variation in time period had no striking influence upon the results.

The sex of the animal is of no significance provided the female is in anestrus. Rogoff and Stewart (22) were the first to demonstrate that the bitch, if adrenalectomized while in heat, could survive as long as 65 days. This observation has been confirmed in this laboratory by withdrawing extract from adrenalectomized bitches when in heat.

On completing the above assay five of the dogs were used to recheck the results. The check assays, Table I, Series B, show the results when one or the other stock diet was fed exclusively. These animals would eat either ration with equal readiness when in good physical condition. Dogs coming into insufficiency and subsisting on Diet B alone usually show an aversion to food earlier than when receiving Diet K. In Series A the animals were started upon the former and gradually worked over to the latter. (This is the feeding schedule in routine assay work.) Inspection of the data, Series B, shows that there was no striking difference in results obtained on the same dog when using the different diets. With Dog 12, who was fed a ration of beef heart, the failing dose was 0.4 gm. in contrast to 0.2 gm. on Diet K. The animal did not relish the ration, and refused the food for the last 5 days of the assay. At the time of realimentation it exhibited no asthenia (spasticity), but did show a continued refusal to eat this diet, somewhat lessened activity, and a markedly elevated blood urea and pulse. The experiments on fasting, Table III, indicate that this early failure of Dog 12 can be accounted for by the voluntary fast, and not the beef heart diet, *per se*.

The check assays on Dogs 23 and 25 indicate that a loss of weight is not necessarily an integral part of the syndrome of cortical insufficiency in the dog. Dog 25 died at a peak weight. Autopsy findings were typical of death from insufficiency. This dog as in some others when subsisting on a dose approximating the minimum maintenance dose always exhibited a fluctuating blood urea, occurring without concomitant changes in clinical condition. Similar fluctuations in blood urea occurred in Dog 20, when held for a period of 33 days upon a minimum maintenance dose of 0.4 gm. It should be emphasized that all blood samples were obtained at least 15 hours after all food had been withdrawn. The possibility remains that these variations of blood urea in animals on the verge of insufficiency may be due in part to a delayed absorption. Food records

for these periods show some correlation between food ingested and blood urea. Whenever an animal took very little food for a day or two, a marked rise in blood urea would occur—being followed by a drop when the ration was again ingested. The great variability in the appetite of these dogs chronically at a level of insufficiency remains unexplained.

TABLE II

*Check Assays on Series of Adrenalectomized Dogs at Various Intervals*

Dog No.	Date of operation	Dosage prior to assay*	1st day of assay	Extract No.	Minimum maintenance dose	Insufficiency between check assays	Time interval†
		gm. gland per kg. per day			gm. gland per kg. per day		days
7	Aug. 31, 1931	15.0‡	Sept. 23, 1931	813-A	3.0		
		20.0‡	Apr. 6, 1932		<2.3	3	164
		6.8	May 3, 1932	31632	0.34		
		8.0	Nov. 21, 1932		0.4	4	165
		10.0	Sept. 2, 1933		0.34	9	276
12	Sept. 30, 1931	10.0	Jan. 26, 1932	1532-B	0.5		
		0 4§	Mar. 7, 1933		0.5	7	359
23	Jan. 11, 1932	8.0	May 3, 1932	31632	0.5		
		7.2	Apr. 14, 1933		0.4	6	289
42	May 19, 1932	4.0	June 11, 1932	D-218	2.0		
		0.75§	Dec. 12, 1932		2.0	5	166

\* Unassayed stock extract. Estimated that 0.5 gm. represents 1 dog unit.

† Number of days which elapsed between the termination of an assay and the beginning of the check assay.

‡ Dissected cortex. Estimated that 5.0 gm. represent 1 dog unit.

§ Previously assayed. Amount indicated known to represent 1 dog unit.

In Table II are tabulated data on several check assays conducted on the same dog at intervals of 164 to 359 days, the same sample of extract<sup>10</sup> being used in each instance. During this interval the dogs experienced from three to seven periods of insufficiency. Comparable findings on the minimum maintenance dose were obtained

<sup>10</sup> Preserved with benzoic acid (0.1 per cent) and stored in the refrigerator at 6°.

in all cases with the exception of the first check assay on Dog 7. The sample of extract was exhausted before the check assay could be completed. One point of particular interest brought out in the assays on Dogs 12 and 42, Table II, is that the minimum maintenance dose is quite independent of the dosage level on which the dog was maintained prior to assay. Dog 12 was receiving an estimated daily dose of 8 dog units per kilo prior to the first and 1 dog unit per kilo for a period of 7 days prior to the check assay. It has been found that the dosage level prior to complete withdrawal of extract has no striking influence on the length of the survival period. Since apparently the hormone cannot be stored and since an overdosage effect of the hormone seemingly cannot be demonstrated, it would appear that all excess of injected hormone over and above the animal's immediate requirement is either excreted or further metabolized.

Experiment shows that cortical hormone added to normal dog urine can be recovered quantitatively. When a massive dose (2340 dog units) of cortical hormone was injected subcutaneously into a normal male dog, less than 200 dog units could be detected in the urine collected for the following 53 hours. The possibility remains that a certain amount of hormone may be excreted by the kidney if large quantities are administered intravenously. The return from insufficiency to normal condition, based on blood urea figures, was about equally rapid by either subcutaneous or intravenous therapy, usually requiring 24 to 72 hours with the doses employed.

Britton, Flippin, and Silvette (23) from their work on adrenalectomized cats expressed the opinion that the oral administration of the cortical hormone held out promising clinical possibilities. Comparative assay findings on the dog demonstrate that in this species the subcutaneous administration of the hormone is more than 12.5 times as efficient as the oral route. The minimum maintenance dose (subcutaneous) was determined on Dog 42 as 0.4 gm. This dog came promptly (7 days) into the state of insufficiency when treated orally with the same extract at a dosage level of 5 gm. (extract administered in gelatin capsules).

It would seem that the therapeutic method of choice would consist in small frequent subcutaneous injections except in emergency when the intravenous route would be indicated.

*Hormone Requirement As Influenced by Environmental Factors—*

The early failure of Dog 12 (Table I) on a beef heart diet, a ration which the animal did not relish, suggested that the nature of the diet as reflected in the voluntary fasting of the test animal may influence assay results. The data recorded in Table III demonstrate the effect of fasting on the hormone requirement. It is of particular interest to note (Period 2) that the dog while on one minimum maintenance dose was thrown into mild insufficiency by withdrawal of food but that the symptoms of insufficiency were readily alleviated by allowing the animal to eat. During 5 days fasting the blood urea became elevated as did also the pulse rate and hemoglobin. The blood sugar dropped to a fasting level. During the period of fasting the water intake was only about 10 per cent of the intake during the control period. The urea, glucose, and hemoglobin returned to the original levels during the post-fasting period. During the period of fasting, on excess dosage of hormone (Period 1), the urea nitrogen level remained in the normal range, the glucose dropped to a fasting level, the pulse rate did not change significantly, while the hemoglobin level rose 29 per cent. In Period 3 the animal was given water by stomach sound in an amount comparable to the total daily water intake during the control period. The hemoglobin and pulse rate became elevated but the urea level remained in the normal range. After a week of fasting with the administration of water by sound the animal failed to recover promptly on refeeding. It seemed to be on the verge of insufficiency judging from the slightly elevated urea and hemoglobin levels even though it was eating well and gaining in weight. On the morning of January 30 the dog was found in a state of collapse. The blood sugar was 45 mg. per cent. It recovered promptly with the treatment indicated. In Period 4 it is seen that orally administered sodium chloride with the accompanying voluntary water ingestion maintained the blood urea and hemoglobin at the control levels during the 7 day period of fasting. During this interval the animal was receiving one minimum maintenance dose of hormone. Two other adrenalectomized dogs, Dogs 7 and 23, were used in these studies on fasting. They reacted in essentially the same manner as Dog 12, except as regards the results obtained on forcing water during fasting (the amount of water used being comparable to the quantity normally

TABLE III

*Effect of Fasting and Ingestion of Water or Salt during Fast on Adrenalectomized Dog Subsisting on Minimum Maintenance Dose of Cortical Hormone*

Dog No.	Date	Body weight	Food offered*	Total water intake†	Urea N	Glucose	Hb	Pulse	Dosage per kilo per day‡	Remarks
Period 1. Effect of fasting while receiving 10 minimum maintenance doses										
12	10-11-32	11.2	0	615(4)	19	84	13.5	72	8.0§	Clinically normal throughout period
	10-18-32	9.6	+	95(7)	23	69	17.4	52		
	10-25-32	11.0	+	660(7)	25	84	13.5	68		
Period 2. Effect of fasting and refeeding while receiving 1 minimum maintenance dose										
	11-21-32	11.0	+		28	93	11.0	80	0.4	Clinically normal throughout period
	11-25-32	11.0	0	690(20)	29		11.3	80		
	11-30-32	9.9	+	70(5)	50	78	16.8	152		
	12-1-32	10.2	+		36	89	15.8	124		Dosage level raised 25 per cent
	12-10-32	11.0	+	800(10)	34	84	14.2	76	0.5	
	12-20-32	11.6	+	795(10)	31	87	11.3	76		
Period 3. Effect of administration of water by sound during fasting while receiving 1 minimum maintenance dose										
	12-29-32	11.6	+	580(9)	18	81	11.7	72	0.4	Clinically normal until noted below
	1-2-33	11.7	0	670(3)	21	91	12.0	88		
	1-9-33	10.4	+	960(7)¶	21	78	16.5	132		
	1-19-33	11.0	+	1015(10)	33		14.5	96	0.5	Distilled water by sound   Dosage level raised 25 per cent Found 9 a.m. in collapsed state¶
	1-30-33	11.3	+		30	45	16.2	172		
	2-14-33	11.1	+		14	83	10.0	72		
Period 4. Effect of salt ingestion during fasting while receiving 1 minimum maintenance dose										
	2-14-33	11.1	+		14	83	10.0	72	0.4	Clinically normal throughout period NaCl administered**
	2-17-33	11.0	0	505(2)	18	84	10.4	76		
	2-24-33	10.2	+	382(7)**	17	51	10.4	48		
	2-25-33	10.4	+		24	87	10.2	56		
	2-27-33	10.4	+	655(3)	27	82	10.1	80		

TABLE III—Continued

Dog No.	Date	Body weight	Food offered*	Total water intake†	Urea N	Glucose	Hb	Pulse	Dosage per kilo per day‡	Remarks
Effect of administration of water by sound during fasting while receiving 1 minimum maintenance dose										
7	12-29-32	10.8	+		17	85	11.2	116	0.4	Clinically normal
	1- 2-33	10.5	0		24	100	14.3	144		Water (900 cc.)†† daily until Jan. 7, 1933
	1- 6-33	10.2	0		19	93	16.2	112		Convulsions‡‡
	1- 7-33	10.1			22	99	17.3	204		Asthenia§§
	1-23-33	10.9	+		18	82	7.9	96		Clinically normal
	2-20-33	11.3	+		16	93	7.9	88	0.4	" "
	2-23-33	11.1	0		23	101	8.9	80		Water (900 cc.)†† daily until Feb. 29, 1933
	2-28-33	10.7	0		21	92	9.8	96		Convulsions
	3- 1-33		+							Asthenic
	3-23-33	11.2	+		20	84	7.2	108		Clinically normal

\* 0 in the food column indicates the complete withdrawal of the food on that date. The fasting period continued until the following entry and was terminated with one-half ration of food. On Jan. 7, 1933 food was offered Dog 7 but none was taken.

† The average total daily intake (water ingested plus water in food consumed) for the number of days (in parentheses) prior to the entry date.

‡ Dosage levels stated were continued through the experimental period indicated or until a different dosage level was adopted in the same experimental period with exceptions noted.

§ Extract 1532-N previously assayed on this dog. 8 gm. = 10 dog units.

|| During the 7 days indicated 900 cc. of water were administered by sound in three equal portions daily at 10 a.m., 2 p.m., and 5 p.m.

¶ Unable to walk; intravenous injection of 40 gm.; copious bloody and watery stools; 100 cc. of 50 per cent glucose were administered by mouth in a.m. and p.m. Much improved on the following day. Normal blood and clinical findings 3 days later on dosage level of 20 gm.; then dropped to 10 gm. for remainder of period.

\*\* During the 7 days indicated 6 gm. of sodium chloride were administered orally (in gelatin capsules) in three equal portions daily at 10 a.m., 2 p.m., and 5 p.m.

†† By sound in three equal portions at 10 a.m., 2 p.m., and 5 p.m.



TABLE III—*Concluded*

‡‡ This blood sample obtained at 10.30 p.m. when the animal was found in convulsions. Complete prostration. Intravenous injection of 40 gm.

§§ Intravenous injection of 40 gm. at 9 a.m. Started to take food on Jan. 8, 1933. 40 gm. subcutaneously until Jan. 14, 1933. Recovery gradual. Appeared clinically normal on Jan. 14, 1933. Reduced dosage to 26 gm. on this date for remainder of period.

||| This blood sample obtained at 3.50 p.m. when animal appeared quiet and weak. Found in convulsions at 5 p.m. Hemoglobin 11.9 gm. per cent. Intravenous injection of 40 gm. Dosage of 10 gm. for remainder of period. Clinically normal on Feb. 29, 1933 and for remainder of period.

ingested during control periods). The pertinent data are recorded in Table III. Dog 7 was found in convulsions on the 4th day and Dog 23 on the 5th day of this regimen. The convulsions came on very abruptly. The clinical picture was quite different from that seen in adrenal insufficiency and resembled those described by Rowntree (24) and others as occurring in water intoxication. The amounts of water which brought on these symptoms were very small compared to the huge quantities required to produce water intoxication in the normal dog. At the time the convulsions occurred both dogs exhibited normal urea and glucose levels, whereas the hemoglobin was elevated in both instances. Both animals recovered with large doses of cortical hormone. These results indicate that the increased hormone requirement is apparently more directly concerned with the failure of the animal to ingest the salts contained in the usual ration. The observations of Loeb, Atchley, Benedict, and Leland (25) and Harrop, Soffer, Ellsworth, and Trescher (26) on the base balance of the adrenalectomized dog seem to offer the explanation for these findings. These studies on fasting emphasize the importance of a diet in assay work which is relished by the test animals in order to avoid the introduction of errors due to voluntary fasting.

It is a common observation that adrenalectomized animals are very sensitive to infection. Assay data are of little or no significance unless the animal is free of all infective processes. This is more important than having the animal in optimal nutritive condition. For example, a dog suffering from a mild respiratory infection had a minimum maintenance dose of at least 4 gm. After complete recovery repetition of the assay showed the minimum maintenance dose to be 0.5 gm., a difference of 800 per cent. In

cases of more severe respiratory infection relatively huge doses (10 to 40 dog units per kilo) are necessary to keep the blood urea at a normal level. Animals can die of these infections with a normal blood urea.

Dogs used for assay work were kept free of intestinal parasites. They were treated with a vermifuge<sup>11</sup> before the second operation. Completely adrenalectomized dogs when treated with a vermifuge require larger amounts of hormone. For example, Dog 11, receiving ten or more minimum maintenance doses daily was clinically normal with a blood urea of 18 mg. per cent. The day following treatment the urea was 50 mg. per cent and marked asthenia developed. On the next day the urea was 22 and there was definite clinical improvement. Similar effects can be demonstrated after  $\frac{1}{2}$  hour of morphine-ether anesthesia. The validity of assay data collected on animals which are being subjected to any abnormal strain would be obviously open to question.

In using the elevation of blood urea alone as a guide in assay work sufficient control data should be available to demonstrate the absence of nitrogen retention due to causes other than adrenal insufficiency. In a normal dog that developed spontaneous nephritis (following a severe distemper) prior to adrenalectomy the urea rose from a previous normal of 25 to a value averaging 47 mg. per cent. In assays conducted with this animal (and controlled by simultaneous assays on two other dogs) the results checked very well when compared on the basis of clinical failure. No comparison could be made on the basis of blood urea alone. The clinical picture of insufficiency was not noticeably influenced by the previous kidney damage.

*Remarks on Method of Assay*—In early assay work (2) the end-point of the assay consisted in a rise in blood urea nitrogen or non-protein nitrogen of 15 mg. or more, accompanied by a loss in body weight, and clinical failure as evidenced by asthenia, vomiting, etc. As a result of the comparative studies reported in this communication the end-point of the assay as now being utilized in this laboratory consists of a rise in blood urea nitrogen of 100 per cent. There may or may not be a weight loss. When it occurs it seldom exceeds 10 per cent of the body weight. Definite

<sup>11</sup> Tetrachloroethylene (Nema worm capsules, Parke, Davis and Company) followed with 10 gm. of magnesium sulfate.

asthenia is encountered only rarely. A somewhat lessened activity and a failing appetite of varying degree are usually the only clinical manifestations of abnormality. The former is usually apparent only to those familiar with the behavior of the test animal. In a series of test animals the percentage variation in the minimum maintenance dose based on clinical failure was less than the variation encountered in the minimum maintenance dose based on a 100 per cent blood urea rise. The somewhat greater degree of accuracy, however, that may be attained by the former method is more than outweighed by the decrease in the risk of losing the test animal in the latter procedure as well as by the saving in time in reconditioning the animals for further assay work. The accuracy of the method is limited both by the differences encountered in the hormone requirement of individual animals and by the magnitude of the differences between successive dosage levels employed in the assay. The error due to these variables has been kept at a minimum by employing the same dogs and a uniform gradation of dose in assaying a series of fractions. Experience in conducting assays during the past few years under the above conditions indicates the error of determination to be approximately  $\pm 25$  per cent. In extraction and fractionation studies two animals are usually employed in ascertaining the potency of any given sample.

#### SUMMARY

The cortical requirement of a series of nine adult adrenalectomized dogs was determined. The individual requirement was found to vary by approximately 100 per cent. In check assays conducted under presumably optimal conditions the requirement of the same animal was found to vary about 25 per cent. The hormone requirement was found to be independent of the time period which elapsed between adrenalectomy and assay. In the adult dog the hormone requirement remains relatively constant over as long a time period as studied (16 months). It is not influenced by sex provided the female is in anestrus; nor is it influenced by the level of hormone dosage prior to the assay. The number of insufficiency periods which the adrenalectomized animal has experienced does not affect the assay results. By adjusting the level of hormone therapy adrenalectomized dogs were maintained in chronic insufficiency for periods of 30 to 40 days.

The cortical hormone requirement of the dog is increased in fasting. The increased requirement is apparently concerned with the failure of the animal to ingest the salts contained in the ration. The diet used in assay work should be relished by the test animals in order to avoid errors due to voluntary fasting.

During fasting the adrenalectomized dog subsisting on a minimum maintenance dose of hormone is sensitive to relatively small amounts of water administered by sound. A clinical condition resembling water intoxication was produced in two of three animals studied.

Orally administered hormone is less than 8 per cent as efficient as when subcutaneously administered.

Cortical hormone injected subcutaneously is not excreted in the urine by the normal dog. Since there is no demonstrable storage in the organism of the dog the hormone is either metabolized or excreted by some other route.

Three adrenalectomized dogs have been maintained on cortical extracts for a period of more than 2 years.

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## PHOSPHATASE STUDIES

### VII. INORGANIC PHOSPHORUS AND PHOSPHATASE OF THE SERUM IN NEW BORN PUPPIES

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The normal serum phosphatase and inorganic phosphorus values in the mature 3 year-old dog (about  $1.5 \pm 0.5$  units and about 3 mg. per 100 cc., respectively) represent a gradual decrease from higher values to be found in well nourished puppies about 2 weeks old: 8 to 10 mg. of inorganic phosphorus and about 10 to 15 units of phosphatase per 100 cc. of serum (1).

Preliminary studies indicated that these latter values represented, in turn, a great and rapid decrease from figures to be found in new born puppies, which were of a very much higher order and varied from litter to litter and sometimes within the litter. The number and condition of puppies in these litters are noted because of their apparent significance in relation to the level of serum phosphatase: 161 and 85 units, respectively, per 100 cc. were found in the two puppies of Litter 17 about 24 hours after birth (about 10 times the average value for young puppies). About 36 hours after the birth of eight puppies of Litter 20, for one puppy, a runt, about 28 units were found, and for the others  $46 \pm 3$  units per 100 cc.;<sup>1</sup> for Litter 21 (four puppies) there were about 30 units per 100 cc. 165 and 136 units, respectively, were found 6 hours after birth in Litter 30 (two puppies).

The serum was barely translucent or quite opaque when the serum phosphatase was highest. The alimentary lipemia indicated that high serum phosphatase was related in the new-born,

<sup>1</sup> The serum phosphatase values for Litters 17 and 20 were reported before the Twenty-sixth meeting of the American Society of Biological Chemists at Philadelphia, 1932 (2).

as in several other feeding experiments, to active food absorption, rather than to processes associated with metabolism of bone (2). It seemed desirable to study the changes of serum inorganic phosphorus and serum phosphatase at successive short intervals after birth, as well as to verify the rôle of feeding in these early changes.

#### *Plan of Experiments*

Three litters of puppies were employed. Litter 1 contained only two animals, Litter 2 seven, Litter 3 nine; the size of Litters 2 and 3 permitted the employment of a total of eight fasting controls. Six controls were removed from the bitches immediately after birth, and before they could nurse; as a further check upon the effect of fasting, two puppies of Litter 3 were removed while nursing, 1 hour after birth. All controls were given water by means of a pipette; one puppy (Litter 2) was allowed to nurse after 8 hours, the others after 15 to 18 hours. All puppies were bled at intervals of about 3 hours during the first 18 hours, twice during the next 30 hours, and less frequently thereafter.

#### *Methods*

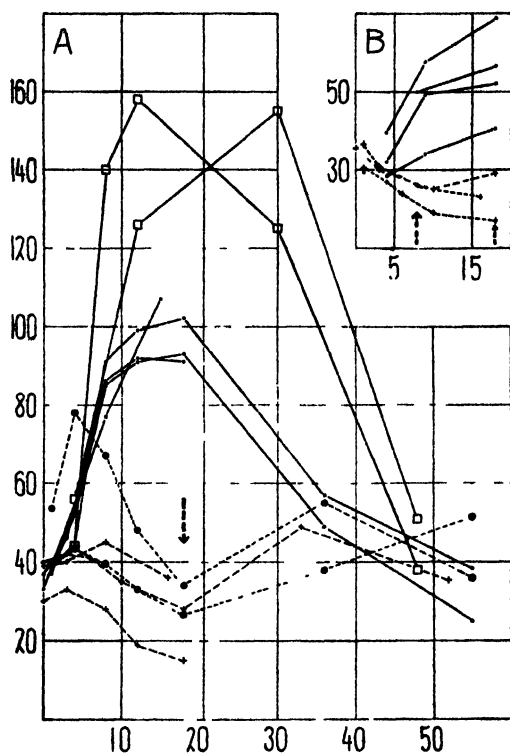
Blood was drawn from the jugular vein and centrifuged after the clot had formed. In view of the frequency of the bleedings during the first 18 hours, just enough blood was drawn for the serum inorganic phosphorus and phosphatase determinations. The various degrees of alimentary lipemia were estimated by the turbidity of the serum, as seen in the centrifuge tube by transmitted light immediately after centrifuging. The sera, when not clear, were designated as opaque, translucent, cloudy, or hazy. This differentiation, while rough, proved satisfactory for our purposes.

Serum inorganic phosphorus and serum phosphatase were determined by methods previously described (3, 4).

#### *Results*

Our phosphatase figures for the period during which they showed the most striking variations are shown in Fig. 1. Later data, as well as observations of seemingly significant serum inorganic phosphorus changes and estimates of lipemia, are included in Table I.

**Fed Puppies**—Serum phosphatase rose from between 30 and 50 units per 100 cc., found during the first 4 hours of life, to as high as 160 units during the first 30 hours. The trend of the curves



**FIG. 1, A AND B.** Serum phosphatase in new born puppies, as affected by fasting and different levels of feeding. In Fig. 1, A the squares distinguish values for the abundantly fed Litter 1 from those for nine puppies of Litter 3; circumscribed crosses, two puppies of Litter 3 nursed during the 1st hour after birth. Fig. 1, B contains curves for Litter 2. The ordinates represent phosphatase units per 100 cc.; abscissae, hours after birth. The solid lines denote the values for puppies nursed throughout; broken lines, fasted puppies; dots and dashes, one puppy of Litter 2 fasted for 8 hours. Arrows indicate the end of the fast.

indicates that the maxima were reached between the 15th and 24th hours after birth. Thereafter serum phosphatase declined rapidly.



TABLE 1.—Serum Inorganic Phosphorus and Serum Phosphatase at Stated Intervals after Birth

Puppy No	Hrs. after birth														Days after birth													
	0		1		4		8		12		18		30		2		3		10		20		30		45			
	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E	P	E		
Litter 2, fed puppies																												
3					8 8h	28 4	7 7h	33 9			7 9h	40 5					5.00	13 2	8 5h	6 4	8 5	4 1	7 5	9 6	9 2	13 1		
4					9 0h	32 0	8 3h	49 1			7 8h	52 2					6.3	11 1	8 7h	6 8	13 1	3 9						
5					8 2	39 7	7 2h	57 5			7 2h	68 7					4.50	17 5	8 0h	8 6	7 2h	5 5	8 0	7 7	9 8	12 7		
6							7 3h	49 9			9 5	56 4					5.50	17 1	9 0c	7 5	7 7h	8 3	8 6	8 7	10 1	11 7		
Litter 2, fasted controls																												
1			8 8	30 4	7 3	26 8	7 6	23 7	7 8 <sub>10</sub>	18 9 <sub>10</sub>	7 6	16 9					8 9h	15 3	7 4c		8 7o	5 0	7 0h	8 0	8 8	7 1		
2	8 3	35 7			7 8 <sub>1</sub>	30 2 <sub>1</sub>	End of fast		7 6 <sub>11</sub>	25 0 <sub>11</sub>	8 4h	29 2					11.2c	15 0	9 6c	7 3	8 5c	6 9	7 7h	11 4	10 7	13 1		
7			7 5	36 1	7 2 <sub>1</sub>	30 8 <sub>1</sub>	7 7 0	26 2			7 5 <sub>1</sub>	23 2 <sub>1</sub>					9 0h	21 9	8 7c	8 8	7 4c	6 3	8 2	9 9	11 0	11 4		
Litter 3, fed puppies																												
1			8 3	37 1	6 8c	52 2	5 8h	85 0	5 0c	91 0	5 0c	93 0	5 1o	49 8	4 7h	24 9												
2			8 0	38 6	6 6h	44 3	5 8o	91 0	6 11	99 0	6 7o	102 0	6 0o	56 7	5 8o	38 4	5 5o	29 2 <sub>1</sub>	9 8o	6 7	8 0c	10 7						
5	9 3	36 8			7 9c <sub>1</sub>	45 9 <sub>1</sub>	5 8h	86 0	5 8c	92 0	6 6o	91 0																
8	9 3	33 3					6 9h	77 0			6 0 <sub>11</sub>	107 0 <sub>11</sub>																
Litter 3, fasted controls																												
3			7 6	38 6	6 9	43 5	6 9	39 5	7 3	32 7	7 8	26 6	7 2o	38 2	6 0o	51 4												
4			8 0	53 5	6 0	78 0	6 8	67 0	7 7	48 3	8 6	34 2	7 8o	55 2	5 0o	38 0	6 7o	19 0 <sub>1</sub>	7 1c	13 8	8 0c	9 0	9 4	12 0				
7	9 0	40 0			7 7 0 <sub>1</sub>	41 6 <sub>1</sub>	7 7 <sub>1</sub>	40 7 <sub>1</sub>	7 2 <sub>10</sub>	34 8 <sub>10</sub>	8 1	28 0	6 9 <sub>1</sub>	49 0	7 6 <sub>1</sub>	35 4	8 0o	17 6 <sub>1</sub>	8 1c	12 2	8 5c	14 2	9 0	10 9				
9	10 0	39 0			7 7 0 <sub>1</sub>	40 0 <sub>1</sub>	7 2	45 0			7 7 <sub>18</sub>	36 3 <sub>1</sub>																
6	9 0	30 0			7 7 0 <sub>1</sub>	33 0 <sub>1</sub>	7 2	28 0	7 2	18 7	8 0	14 7																

The fast began at birth in all cases except for Puppies 3 and 4 of Litter 3 (1 hour after birth); fast ended 18 hours after birth, with one exception, Puppy 2 of Litter 2. P, serum inorganic phosphorus, mg per 100 cc.; E, serum phosphatase, units per 100 cc serum. ? , estimated value; accurate determination impossible for technical reasons. Inferior figures show the actual time at which blood was drawn, when it differed from the time stated at the top of the column.

The early rise of serum phosphatase was as great in Litter 1 and almost as great in Litter 3 of the present series as it had been in Litters 17 and 30. In Litter 2 a smaller rise occurred; this litter, consisting of seven puppies, was the first litter of a young dog, in which lactation was not as vigorous as in the other females;

the serum, during the early intervals was at most hazy; about 9 days later, their serum phosphatase had declined to an unusually low figure; these puppies gained at a slower rate than other litters, until supplementary food could be given, after which they gained satisfactorily, the serum phosphatase also rising.

Rough estimates of lipemia revealed certain correlations with serum phosphatase. Up to about 4 hours after birth the serum was clear, or at most slightly hazy. Later, when a relatively slow rise to moderately high serum phosphatase (average, about 55 units per 100 cc.) was observed in Litter 2, the sera were hazy; at the same intervals after birth the sera in Litters 1 and 3, showing a more rapid rise of phosphatase and higher maxima (averages about 155 and 95 units, respectively), were translucent or opaque with fat. Comparisons between individual puppies within Litter 2 showed no definite correlation between apparent lipemia and maximum serum phosphatase; the highest maxima (68.7 and 56.4, Puppies 5 and 6, Table I), were reached in sera which were at most slightly hazy, while somewhat more turbid sera showed relatively lower maxima (40.5 and 52.2, Puppies 3 and 4, Table I). In Litter 3 the maxima were within a relatively narrower range (about 90 to 110 units); when these maxima were observed, the sera of three puppies were translucent or opaque; the serum of the fourth was cloudy. The increase of serum phosphatase between successive analyses was not associated with consistent increase of turbidity in Litter 2; in Litter 3 the increase of turbidity was consistent, this correlation, however, being maintained only during the 1st day after birth. The decrease of serum phosphatase that followed its early rise was not associated with a diminution of alimentary lipemia; in Litter 2 the lipemia was greater at the 72 hour interval than at any time before, but the serum phosphatase was lower.

Serum *inorganic phosphate* in the well fed Litter 3 declined markedly as the serum phosphatase rose.

*Fasting Controls*—These were used under a variety of conditions in order to vary the demonstration of the effect of feeding. In Litter 2, two puppies fasted for 16 and 18 hours, respectively, showed a continuous decline of serum phosphatase; one, fasted for 8 hours, showed declining phosphatase until 10 hours after birth; a slight phosphatase rise, which followed, was obviously due to discontinuance of the fast. The indications thus obtained were tested more fully in Litter 3. Two of the five fasting controls had been allowed to nurse for an hour immediately after birth, and serum phosphatase rose slightly in one and very markedly in the other during the next 3 hours; in two puppies, fasting from birth,

serum phosphatase rose slightly for 3 hours, and in one for 8 hours after birth. After these initial increases serum phosphatase declined in all five puppies until the end of the fast. About 18 hours after the termination of the fast serum phosphatase had increased in the three controls that were continued in the experiment; during the following 18 hours the rise continued in one, while in two the phosphatase decline had begun.

The serum remained clear during the entire period of fasting; it became hazy in puppies of Litter 2, and opaque or translucent in puppies of Litter 3 after they had nursed. There was no definite correlation between the degree of lipemia and the phosphatase rise after termination of the fast.

The serum *inorganic phosphorus* decreased during the fast in Litter 3, but not as markedly as in the fed puppies of the same litter; after its termination serum inorganic phosphorus fell further, indicating an effect similar to that observed in the fed puppies.

#### DISCUSSION

The occurrence and significance of phosphatase were reviewed recently by Robison and Kay (5, 6). We discussed in a recent paper the possible rôle of the intestinal mucosa, liver, kidney, and muscle as non-osseous sources of increased serum phosphatase, which was associated with hyperglycemia and hypophosphatemia after ingestion of glucose and dextrin (7). In the present experiments the causes of the rise of serum phosphatase in new born nursing puppies are similar but less specific, inasmuch as milk, a complex food mixture, is involved, rather than a single substance like dextrin or glucose. Our earlier experiments indicated that protein feeding lowered serum phosphatase, while fat affected serum phosphatase but slightly, possibly lowering it (7); we used puppies about 4 months of age. However, the inferences from observations on the older puppies would not necessarily apply to puppies during the first few days after birth, when their reactions to feeding, as well as fasting, seem unique; it is possible that an investigation of the effects of specific food substances (carbohydrates, fats, proteins) separately administered will yield different results than in older puppies. It is not permissible, therefore, to conclude at this time that the serum phosphatase rise in new born puppies is due solely to the carbohydrate in the milk. The decline

of the serum inorganic phosphorus suggests, however, the possibility that the serum phosphatase increase is associated with synthesis of organic phosphorus compounds.

While a rough correlation between lipemia and the serum phosphatase level seems to exist, at least during the first 18 hours, its breakdown thereafter decreases the likelihood that the serum phosphatase level is a function of lipid absorption as such, or of lipid metabolism. In older puppies, it may be noted, ingestion of fat causes no significant serum phosphatase changes.

In mature and even in young dogs a marked decrease of serum phosphatase can be obtained only after very prolonged fasting or malnutrition. The rapid decrease of serum phosphatase in fasted puppies is apparently a phenomenon as peculiarly restricted to the new-born as the phosphatase rise in fed puppies. This decrease may be due to retention by tissues, secretion in the urine, destruction, or inactivation. In any event, if these processes are assumed to be normal and to occur also in nursing puppies, as is probable, then it follows that the total formation of serum phosphatase in the latter is greater than indicated by the increase in comparison with the initial figure. A more accurate estimate of the influence of nursing may be obtained by comparing the nursing and fasting puppies of the same litter. The difference between these was greatest about 18 hours after birth, the serum phosphatase averaging about 95 and 25 units in the nursed and fasting puppies, respectively, of Litter 3, and 55 and 25 units in Litter 2, which was not as well fed.

Another difference between Litters 2 and 3 may be noted: the serum phosphatase declined in fasted puppies of the former litter during the first 3 or 4 hours; in the latter serum phosphatase remained almost constant or rose during the same interval. This early, as well as the subsequent course of the serum phosphatase curves in both fasted and fed new born puppies, indicates the influence not only of the quantity or quality of the feeding, but also of the condition of the puppies at birth,—of what may be termed their “prenatal nutrition” (see also the value for the runt of Litter 20, cited above).

Comparisons of Litters 2 and 3 have provided an excellent, although unpremeditated opportunity to illustrate repeatedly, in their various manifestations, the graded effects of good feeding,

poor feeding, and fasting. A striking illustration of the effects of *abundant feeding* was equally unpremeditated: Litters 17 and 30, as well as Litter 1 of the new series, happened to consist of two puppies each; they were consequently the best nourished, and showed the highest serum phosphatase figures that we observed in new born puppies.

After these experiments were completed, Stearns and Warweg (8) reported plasma phosphatase figures in children. The values, as indicated in their Chart 1 (in terms of Kay's units), ranged between about 0.1 to 0.2 unit per 100 cc. at birth, rose to between about 0.3 to 0.5 unit at 1 month of age, declined to about 0.3 unit at 1 year, about 0.2 unit during childhood, and about 0.15 unit thereafter. It is not stated whether the curves represent results of single determinations on many infants during the first month or two, or successive determinations on fewer infants. We have discussed elsewhere (4) the defects of Kay's procedure, which are seemingly responsible for the low ratio of the plasma phosphatase values in children and adults. However, Stearns and Warweg's relatively high values at about 1 month of age show, within the limitations of the method used, the changes that undoubtedly take place. The time relations may prove to be similar to those found in our puppies, after allowing for the difference in life span or time required to reach maturity, and the significance of both sets of data is probably similar. They give independent evidence of an early rise of serum (or plasma) phosphatase in the new-born. In our experiments controls proved that the normal course of serum phosphatase in the new-born could be reversed by fasting; low plasma phosphatase figures in infants during the first 2 months might conceivably be associated with malnutrition.

Comparison of phosphatase figures, before and after supplementary feeding made good the quantitative deficiencies suffered by the nursing puppies of Litter 2 in this experiment, indicates that the great variability of serum phosphatase in the young is due to variations in the state of nutrition. Similar variations due to the same cause were observed in other litters after the end of the more striking early phenomena in the new-born.

#### SUMMARY AND CONCLUSIONS

1. Serum phosphatase in new born puppies averaged about 40 units per 100 cc. at birth and for about 4 hours thereafter. Only

slight changes were observed in most cases during that period either in fed or fasted puppies in Litter 3 (good prenatal nutrition).

2. Serum phosphatase rose thereafter in nursing puppies as high as 160 units, in small litters, during the first 24 hours, then decreased rapidly for a few days to about 20 units, and more slowly to the normal range of young puppies. Poorly nourished puppies show lower phosphatase figures at every stage.

3. Fasted puppies showed a rapid, continuous, and marked decline of serum phosphatase during the period of the fast (in Litter 2—poor prenatal nutrition—the decline began before the 4 hour interval); after the end of the fast, serum phosphatase rose for 18 to 36 hours; the normal downward trend followed.

4. The highest serum phosphatase figures were observed in well fed puppies. They were associated with a fall of inorganic serum phosphorus and with evidence of lipemia during the first 18 hours. The correlation between lipemia and serum phosphatase level did not hold later. Both may be considered different manifestations of active absorption; the serum phosphatase rise is, however, peculiar to the first 24 hours of life.

5. 18 hours after birth the serum phosphatase figures averaged about 25 units in fasting controls, about 55 units in poorly fed puppies, and about 95 in well fed puppies. These figures indicate the relative effects of different levels of nutrition in the new-born.

6. The curves of serum phosphatase in nursing and fasting new born puppies give additional evidence of non-osseous origin of serum phosphatase. Under the special conditions of nutrition in the new-born the contributions of the intestinal mucosa and of the liver to the serum phosphatase are particularly plausible.

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## THE INDUCED OXIDATION OF CYANIDE\*

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In the studies in which cyanides have been employed to inhibit metal catalysis generally the possibilities have been overlooked that the oxidation of cyanide itself might be induced or that the latent reducing capacity of some component of the system might be activated by the cyanide. In so far as either of these processes occurs, the interpretation of the effect of cyanide on oxygen consumption is greatly complicated.

We have observed that oxygenation of alkaline glucose systems containing cyanide provides a relatively simple demonstration of the induced oxidation of cyanide as well as its activating effect. Our data demonstrate that optimum concentrations of cyanide will increase by 60 per cent the oxygen absorption of glucose in 0.5 N NaOH, and result in the induced oxidation of 0.50 to 0.75 mm of cyanide per mm of glucose oxidized.

Approximately 50 per cent of the *extra* oxygen is used in the induced oxidation of the cyanide; the remaining 50 per cent in a more complete oxidation of the sugar. The cyanide is converted chiefly into oxalic acid and ammonia, while glucose forms more carbon dioxide in the presence of cyanide.

The literature contains many instances of increased activity in the presence of cyanide, only a few of which may be mentioned. Benedict (1922) employed cyanide activation for the determina-

\* Part of the data in this paper is taken from a dissertation presented by Charles J. Deere to the Committee on Graduate Study in partial fulfillment of the requirements for the degree of Master of Science, University of Tennessee, September, 1932.

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tion of uric acid. The Ariyama (1928) method for glyoxal depends upon the same principle. Hyman (1916) found a 40 per cent increase in the oxygen consumption of sponges in the presence of  $5 \times 10^{-6}$  M KCN. An increase in the oxygen consumption and carbon dioxide production of potato tubers in an atmosphere containing HCN has been reported by Hanes and Baker (1931). Slight increases in the rate of respiration of *Paramecia* resulting from cyanide have been recorded by Shoup and Boykin (1931). Wright and Van Alstyne (1931) observed that cyanide-hematin was an oxidation catalyst for linseed oil, comparing favorably with pyridine-hematin. Some of the accelerated oxidations reported in the literature are due possibly to uncontrolled pH effects; however, the examples cited here, and perhaps many others, appear to result from definite cyanide effects. Ellinger (1924) observed that on treating frog muscle, pH 7.2, with increasing concentrations of cyanide there appeared a diminution in oxygen consumption followed by an augmentation. With high concentrations of cyanide (90 mm per liter) the oxygen consumption in some instances exceeded that of the original unpoisoned hashes. Ellinger interpreted his results as indicating the induced oxidation of cyanide. Since his data consist only of oxygen absorption values, obviously they may be interpreted also as a result of activation by cyanide or a combination of this and induced oxidation. Dixon and Elliot (1929) recognized the possibility of the induced oxidation of cyanide in their work on tissue respiration but neglected its possible activating effects.

We do not imply that our results with glucose-alkali-cyanide systems have a counterpart in tissues, but certain similarities between the behavior of glucose in the two systems and more especially the stimulating effects of cyanide cited above make it necessary to consider that possibility.

### Methods

*Oxygen Absorption*—The Warburg manometric technique was employed for small quantities not chemically analyzed subsequently. For quantities suitable for analysis of products, as well as oxygen absorption, a macromethod described by Shaffer and Harned (1931) was used.

*Total Base*—The original total base in all experiments was 0.5 N.



This consisted of the alkalinity of the NaCN (considered as NaOH) plus the requisite amount of NaOH.

*Glucose*—An aliquot of the glucose-cyanide-alkali mixture was measured into a volumetric flask, acidified with sulfuric acid, aerated to remove the cyanide, neutralized to phenolphthalein with NaOH and diluted to volume. The glucose content of this solution was determined by the Shaffer-Hartmann-Somogyi method.<sup>1</sup>

*Cyanide*—After the preliminary ammonia aeration of glucose-cyanide mixtures, a 5 cc. aliquot was measured into a 25 × 200 mm. Pyrex test-tube. This was diluted with 10 cc. of water and connected for aeration to a 100 cc. graduated receiver containing 25 cc. of 0.02 N AgNO<sub>3</sub> and 5 cc. of 2.5 N NaOH. By suction 5 cc. of 5 N H<sub>2</sub>SO<sub>4</sub> were drawn into the tube containing the cyanide and the HCN aerated into the alkaline silver solution. The aeration proceeded 30 minutes, the last 15 minutes at 60°. The receiving solution was then acidified with 2 cc. of concentrated HNO<sub>3</sub>, diluted to volume, and a 50 cc. aliquot titrated with 0.01 N KCNS. A blank determination was made simultaneously.

*Ammonia*—Since the ammonia formed was used to confirm the cyanide lost, it was desirable that both determinations should be made on the same sample. The oxygenation bottles were fitted with 2-hole rubber stoppers bearing sealed capillary tubes. When the oxidation of the sugar in the glucose-cyanide-alkali mixture was complete, the sealed capillary tubes were broken and a rapid current of moist air was drawn through the bottles for 15 minutes, the ammonia being absorbed and estimated colorimetrically. By this procedure the ammonia vapor tension was reduced to a degree that permitted transfer of the solution for the cyanide determination without appreciable loss of ammonia. After the removal of the HCN from 5 cc. aliquots, as described above, the residue was made alkaline with NaOH and the remainder of the ammonia was aerated into HCl and Nesslerized. Appropriate blanks were subtracted. The sum of the preliminary and final determinations calculated in mm per liter is the total ammonia formed.

*Oxalate*—An aliquot of the oxygenated mixture, usually 125 cc.,

<sup>1</sup> This reagent was suggested in a personal communication from Dr. P. A. Shaffer and is identical with Reagent 50 reported by Shaffer and Somogyi (1933).

was acidified with glacial acetic acid, and the cyanide removed by aeration. The residue was neutralized to phenolphthalein with concentrated NaOH, barely acidified with glacial acetic acid, and transferred quantitatively to a 250 cc. centrifuge bottle. Calcium acetate (25 cc. of 30 per cent solution) was added, the solution raised to boiling temperature in a water bath, allowed to cool slowly, and placed in a refrigerator overnight. The precipitate was collected in a König type, porous bottom, Gooch crucible, and washed with ice water until the filtrate was free of calcium. The crucible and contents were immersed in 0.4 N  $\text{H}_2\text{SO}_4$ , heated to 70°, and titrated with permanganate.

*Volatile acids and formic acid* were determined by the procedures described by Shaffer and Friedemann (1924).

*Carbon Dioxide*—An aliquot of the glucose-alkali-cyanide mixture was acidified with  $\text{H}_2\text{SO}_4$  containing  $\text{HgSO}_4$  to retain the cyanide, and the  $\text{CO}_2$  removed by aeration and trapped in a bead tower by standard alkali. The solution was washed from the tower with carbonate-free water,  $\text{BaCl}_2$  was added to precipitate the carbonate, and the residual alkali titrated with 0.1 N HCl to the phenolphthalein end-point. The results were corrected by the proper blanks.

#### EXPERIMENTAL

*Cyanide Oxidation and Its Products*—When it was found that cyanide, instead of inhibiting the oxidation of glucose in alkaline solution by air or  $\text{O}_2$ , markedly increased the  $\text{O}_2$  consumed, this fact seemed to merit further study because of considerations mentioned in the introductory paragraphs. The principle questions are the fate of the cyanide, other products resulting from the increased oxygen consumption, and the probable mechanism of the cyanide effect.

Oxamide was found by Attfield (1863) to result from the oxidation of cyanide by  $\text{H}_2\text{O}_2$ , a reaction later studied by Radziszewski (1885) and Masson (1907). The fact that Shaffer and Harned (1931) demonstrated the formation of peroxide during the autoxidation of alkaline sugar solutions suggested that oxalate might be a product of cyanide oxidation in the presence of glucose. Upon examining oxygenated glucose-alkali-cyanide solutions for oxamide we obtained a slight but definite biuret test even in the

presence of the large quantities of cyanide. The Volhard titration confirmed the loss of cyanide, 50 to 90 per cent of which was recovered as oxalate (Table I).

As shown by the Volhard titration values (Table I) and the oxygen consumption data (Table II) only traces of cyanide disappear when cyanide is oxygenated in the absence of glucose under conditions otherwise identical. In this case no oxalate is formed.

TABLE I  
*Induced Oxidation of Cyanide*

Total alkalinity (NaCN calculated as NaOH) = 0.5 N NaOH, at 37.5°; macromethod.

Experiment No.	Time	Initial glucose	Glucose lost	Initial cyanide	Cya- nide lost*	Am- monia formed*	Oxalic acid formed
	hrs.	mm per l.	mm per l.	mm per l.	mm per l.	mm per l.	m.-eq. per l.
Composite, oxygenated†...	37	0.0		57.8	0.54	0.60	0.0
3. Oxygenated.....	41	29.5	29.3	19.5	6.9	6.3	6.1
Anaerobic.....	41	29.5	27.0	19.5	3.1	1.8	0.0
4. Oxygenated.....	40	29.9	29.8	39.4	11.0	11.4	7.6
Anaerobic.....	40	29.9	26.6	39.4	4.4	4.2	0.0
2. Oxygenated.....	42	29.9	29.9	73.2	14.7	15.4	7.5
Anaerobic.....	42	29.9	27.2	73.2	6.7	5.3	0.0
5. Oxygenated†.....	44	29.5	29.3	76.6	17.4	16.9	9.0
Anaerobic.....	44	29.5	27.1	76.6	6.4	6.3	0.0
1. Oxygenated.....	19	29.4	29.4	80.0	15.2		
Anaerobic.....	19	29.4	25.2	80.0	8.2		
7. Oxygenated.....	19	30.0	29.8	0.0			0.0

\* Corrected by appropriate blank.

† Average of five experiments, concentrations ranging from 20 to 80 mm NaCN per liter.

‡ The temperature during part of the experiment was 41°.

If cyanide is permitted to stand anaerobically with glucose in 0.5 N alkali until the reducing power of the glucose has disappeared, some cyanide is lost; however, the amount is only a fraction of that lost if the mixture is oxygenated. Under anaerobic conditions also no trace of oxalate can be found. Added oxalate may be quantitatively recovered from alkali-cyanide as well as from alkali-glucose-cyanide systems whether they have been incubated under anaerobic or aerobic conditions. With the quan-

tities of glucose employed in our experiments the oxidation by molecular oxygen in the absence of cyanide forms no detectable oxalate. The appearance of oxalate clearly distinguishes the loss of cyanide through induced oxidation from the anaerobic loss through cyanhydrin formation and hydrolysis.

The quantity of cyanide lost through induced oxidation depends not only upon the concentration of glucose but also upon the cy-

TABLE II  
*Influence of NaCN on Oxygen Absorption of Glucose*  
Complete oxidation (22 hours each) at 37.5°; Warburg method.

Initial glucose	Initial NaOH	Initial NaCN	Oxygen absorbed				Average	Average of oxygen absorbed per mm glucose oxidised
mm per l.	mm per l.	mm per l.	milliatoms per l.	milliatoms per l.	milliatoms per l.	milliatoms per l.	milliatoms per l.	milliatoms
5	500	0.00	13.4	13.4	13.6	13.2	13.4	2.68
5	499	1.04	14.8				14.8	2.96
5	495	5.04	17.0	16.7*	17.6	17.1*	17.1	3.42
5	490	9.62	18.1	18.2*	18.9	18.7*	18.5	3.70
5	480	20.00	19.6				19.6	3.92
5	470	30.00	20.6	20.7*			20.7	4.14
5	460	40.00	21.0	21.0*			21.0	4.20
5	440	60.26	21.6	21.3*	21.3	21.5*	21.4	4.28
5	430	69.60	21.5	21.8*	22.0	22.2*	21.9	4.38
5	420	77.20	21.9	22.0*	22.0	21.7*	21.9	4.38
0	495	5.04	0.20*					
0	440	60.00	0.18*					
0	420	80.00	0.06*					

\* The absorbing tubes in the flask contained 0.5 cc. of 0.5 N H<sub>2</sub>SO<sub>4</sub> to absorb ammonia.

anide to glucose ratio. With an initial glucose concentration of 30 mm per liter, the oxidative loss of cyanide was increased from 7 to 15 mm by increasing the initial cyanide concentration from 20 to 80 mm per liter.

*Oxygen Absorption*—More oxygen is absorbed in the presence of cyanide than corresponds to its oxidation, the excess being consumed in a more complete oxidation of the sugar. With 5 mm of glucose per liter (Table III) the presence of 1 mm of cyanide results

in an increase in oxygen absorption of 1.4 milliatoms per liter; of this quantity at most 1 milliatom or 72 per cent could be accounted for by cyanide oxidation. With 30 mm of glucose (Experiment 4, Table I) and 40 mm of cyanide it may be calculated that the *extra* oxygen consumed (over that of glucose in the absence of cyanide) was 24 milliatoms, while only 11 mm of cyanide were oxidized. Since the products of cyanide oxidation account for the consumption of only 1 atom of oxygen per mole of cyanide,

TABLE III

*Relation of Cyanide to Glucose Ratio to "Extra Oxygen"\* Consumption*

Complete oxidation at 37.5°; total alkalinity (NaCN calculated as NaOH) = 0.5 N NaOH.

Initial NaCN	Initial glucose	Ratio NaCN to glucose	Extra oxygen per mm glucose oxidized
<i>mm per l.</i>	<i>mm per l.</i>		<i>milliatoms</i>
1 0†	5.0	0 20	0.28
5.0†	5.0	1.0	0.74
10.0‡	10.0	1.0	0.82
9.6†	5.0	1.9	1.02
20.0‡	10.0	2.0	1.05
30.0‡	10.0	3.0	1.24
20.0†	5.0	4.0	1.24
40.0‡	10.0	4.0	1.29
80.0‡	10.0	8.0	1.49
60.3†	5.0	12.0	1.60
69.6†	5.0	13.9	1.70
77.2†	5.0	15.4	1.70

\* The "extra oxygen" is the difference between the amounts absorbed in the presence and absence of cyanide.

† Warburg method.

‡ Macromethod.

approximately 50 per cent of the *extra* oxygen must be assigned to a more complete sugar oxidation. This indicates that glucose (like glyoxal and uric acid) is activated by cyanide.

Chart I shows the effect of cyanide upon the oxygen consumption of alkaline glucose systems which contain identical concentrations of glucose and total alkali.

A point of interest is the fact that the presence of cyanide, although greatly increasing the amount of oxygen absorbed, does

not alter the specific rate of the oxidations (Harned and Deere, 1932); the latter is determined by the rate of glucose activation by the alkali.

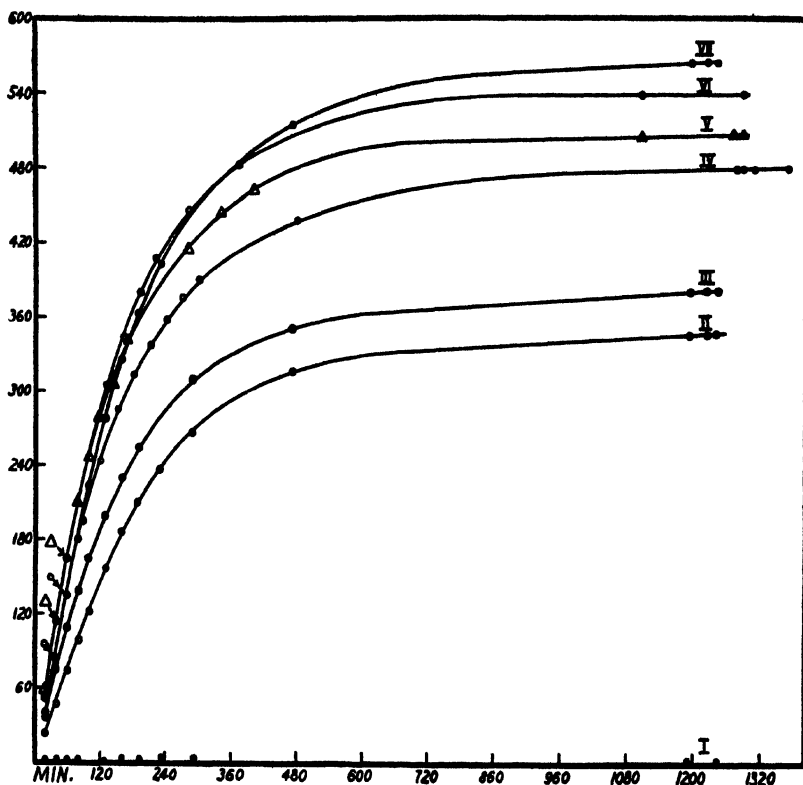


CHART 1. Effect of cyanide on the oxygen consumption of alkaline glucose solutions. Total alkalinity of each solution = 0.5 N ( $\text{NaCN} + \text{NaOH}$ ) at  $37.5^\circ$ . Curve I, no glucose, 80 mm of NaCN; Curve II, 5 mm of glucose, no cyanide; Curve III, 5 mm of glucose, 1 mm of NaCN; Curve IV, 5 mm of glucose, 10 mm of NaCN; Curve V, 5 mm of glucose, 20 mm of NaCN; Curve VI, 5 mm of glucose, 40 mm of NaCN; Curve VII, 5 mm of glucose, 80 mm of NaCN. The ordinate readings show  $\text{O}_2$  consumption in c.mm. per 2.3 cc. of solution.

*Ammonia*—1 mole of ammonia is formed for each mole of cyanide oxidized (Table I). Since ammonia may be formed by hydrolysis of cyanhydrins, cyanate, or oxamide, its appearance gives no evidence as to the nature of the reaction.

**Oxalic Acid**—The oxalic acid obtained is strictly a peroxidation product of the cyanide. We have never failed to obtain it in large quantities when alkali-glucose-cyanide solutions were oxygenated (Table I). On the contrary, the omission of any constituent of the system prevents its formation. The calcium oxalate was precipitated in the presence of acetic acid in order to avoid possible contamination from the calcium salts of sugar acids. The crystalline form of the precipitate was that of calcium oxalate and was further identified as follows: After titrating the solution of the precipitate with permanganate, the calcium was precipitated again as oxalate. The second titration was the same as that of the first precipitate. We also recrystallized some of the precipitate, dried it to constant weight, and found its permanganate titration to be identical with the calculated titration for an equivalent weight of calcium oxalate.

**Formic Acid and Carbon Dioxide**—When the concentration of glucose is 30 mM and cyanide is 20 mM per liter in 0.5 N total alkali, oxygenation yields exactly 1 mM of volatile acid (all of which is formic acid) for each molecule of glucose lost. Glucose under the same conditions except for the absence of cyanide yields the same amount of formic acid. This fact indicates that no part of the formic acid originates from the cyanide.

The  $\text{CO}_2$  produced with the concentrations stated above is equivalent to 0.4 mM per mM of glucose oxidized, while in the absence of cyanide, glucose alone yields only 0.1 mM of  $\text{CO}_2$ . Since oxalate accounts for nearly all of the cyanide disappearing, the increased production of  $\text{CO}_2$  in the glucose system containing cyanide must be derived from glucose. The formation of this  $\text{CO}_2$  roughly corresponds with the surplus of the *extra*  $\text{O}_2$  (over that equivalent to cyanide oxidation) which must be assigned to glucose oxidation.

**Mechanism of Reaction**—We are not able to decide whether the cyanide oxidation results primarily from the oxidation of a cyanhydrin-like complex, or from the oxidation of free cyanide by a sugar peroxide or by hydrogen peroxide; probably all of these reactions occur. Peroxide is known to be formed (Shaffer and Harned, 1931). The behavior of alkaline sugar solutions with cyanide also demonstrates some compound formation. Under anaerobic conditions the brown color of alkaline sugar solutions appears more

rapidly in the presence of cyanide, though, just as without cyanide, browning is prevented by aeration. The continuous addition of hydrogen peroxide over a 6 hour period to a solution of alkali-glucose-cyanide, under nitrogen, does not form oxalate. The hydrogen peroxide was added in small quantities but the total equivalents were 5 times the theoretical  $O_2$  equivalents. Masson's extensive studies (1907) demonstrated that only in acid solution is oxamide a product of cyanide oxidation by hydrogen peroxide. Although our data do not exclude hydrogen peroxide as an intermediary in the  $O_2$  oxidation, they emphasize the existence of another reaction yielding oxalate.

#### SUMMARY

1. The oxidation of sodium cyanide, yielding as the principal products oxalate and  $NH_3$ , is induced by the oxygenation of alkaline glucose solutions.

2. About half of the *extra* oxygen absorbed by the alkaline glucose system under the influence of cyanide is used in the oxidation of the cyanide; the remainder in a more complete oxidation of the sugar.

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# SYNTHESIS OF THE HEXURONIC ACIDS\*

## V. THE SYNTHESIS OF *l*-MANNURONIC ACID FROM *l*-MANNOSACCHARIC ACID

By CARL NIEMANN, ROBERT J. McCUBBIN, AND KARL PAUL LINK

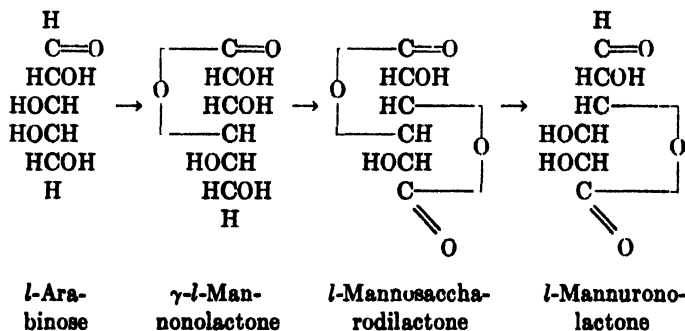
(From the Biochemistry Research Laboratory, Department of Agricultural Chemistry, University of Wisconsin, Madison)

(Received for publication, January 20, 1934)

In 1923 Kiliani (1) reported that  $\gamma$ -*l*-mannonolactone on oxidation with nitric acid yielded *l*-mannuronic acid. The supposed uronic acid was not isolated directly but was characterized as a semicarbazone. 2 years later Kiliani (2) declared that the existence of *l*-mannuronic acid was without foundation since it was learned that the compound isolated was in reality the monosemi-carbazide of *l*-mannosaccharic acid lactone.

Apparently this negation of the synthesis of *l*-mannuronic acid has been overlooked for in three recent treatises (3-5), the synthesis of *l*-mannuronic acid by the oxidation of  $\gamma$ -*l*-mannonolactone is described as having been realized. In this communication we report the synthesis of *l*-mannuronolactone by effecting a partial reduction of *l*-mannosaccharic acid dilactone with sodium amalgam.

The synthesis is illustrated by the following structural formulæ.



\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by a grant from the University Research Fund and the Wisconsin Alumni Research Foundation.

## EXPERIMENTAL

The melting points and rotations were made by using the methods previously described (6). The Pregl micromethods were employed for the analyses.

*Preparation of l-Mannosaccharic Acid Dilactone*—Crystalline *l*-arabinose was treated with hydrogen cyanide according to the procedure described by Fischer (7) and Upson, Sands, and Whitnah (8). After the hydrolysis of the nitriles with barium hydroxide and a subsequent elimination of the barium with sulfuric acid, the aqueous solution was dehydrated with the aid of butyl alcohol (9). The crude product, that crystallized out of the butyl alcoholic solution upon concentration and cooling, was recrystallized from hot 95 per cent ethyl alcohol. The crystalline  $\gamma$ -*d*-mannonolactone obtained melted at 149–150° and showed an  $[\alpha]_D^{20}$  of  $-52.0^\circ$ .

The aldolactone was converted into *l*-mannosaccharic acid dilactone by oxidation with nitric acid, as directed by Kiliani (10). The dilactone obtained from the reaction mixture was recrystallized from 95 per cent ethyl alcohol. It melted at 183–185° and showed an  $[\alpha]_D^{20}$  of  $-202.5^\circ$ .

*Preparation of Barium l-Mannuronate*—15.0 gm. of *l*-mannosaccharic acid dilactone were converted into barium *l*-mannuronate by following the procedure described by Niemann and Link (11) for the preparation of barium *d*-mannuronate. The barium salt prepared by this procedure was contaminated in part with an unknown substance. Of the 12.0 gm. of crude barium salt obtained approximately 30 per cent was in the form of a non-reducing substance.

*Rotation*— $[\alpha]_D^{27} = +4.0^\circ \pm 1^\circ$  (in water,  $c = 2.43$  per cent).

*Analysis*—Calculated for  $(C_6H_7O_7)_2Ba$ . Ba 26.3

Found.

" 24.0, 24.8

*Preparation of l-Mannuronolactone*—10 gm. of the crude barium salt were converted into the lactone as previously described for the corresponding *d*-enantiomorph (11). The first crystalline product isolated was a substance having a melting point of 159–160° and a rotation close to 0. After the removal of this fraction (0.5 gm.) the residual syrup was taken up in absolute alcohol and

acetone. After standing for several weeks the solution turned into a semisolid mass. This product was filtered off and recrystallized from absolute ethyl alcohol, yielding 0.5 gm. of pure crystalline *l*-mannuronolactone.

**Melting Point**—The lactone melted at 143–144° with decomposition.<sup>1</sup>

**Rotation**— $[\alpha]_D^{27} = -92.0^\circ \pm 2^\circ$  (maximum in water,  $c = 0.9$  per cent).

<b>Analysis</b> —Calculated for $C_6H_8O_6$ .	C 40.92,	H 4.55
Found.	" 40.85, 40.87,	" 4.62, 4.60

**Preparation of Brucine *l*-Mannuronate**—1.30 gm. of the crude barium salt were converted into brucine *l*-mannuronate by following the conditions previously given for the preparation of brucine *d*-mannuronate (11). The alkaloidal salt was recrystallized from 95 per cent ethyl alcohol and dried at 78° over phosphorus pentoxide for 10 hours prior to analysis.

**Melting Point**—The salt melted at 155.5–156.5° with decomposition.

**Rotation**— $[\alpha]_D^{27} = -22^\circ \pm 2^\circ$  (in water,  $c = 2.45$  per cent).

<b>Analysis</b> —Calculated for $C_{29}H_{34}O_{11}N_2$ .	OCH <sub>3</sub> 10.55, N 4.76
Found.	" 10.69, " 4.99

**Preparation of Barium *p*-Bromophenylhydrazone-*l*-Mannuronate**—Approximately 1 gm. of the crude barium salt was converted into the hydrazone barium salt by following the procedure described by Niemann, Schoeffel, and Link (6) for the preparation of barium *p*-bromophenylhydrazone-*d*-mannuronate. The method employed is given under the title, Section III, Procedure 1.

<b>Analysis</b> —Calculated for $(C_{12}H_{14}O_6N_2Br)_2Ba$ .	Ba 15.95, N 6.51
Found.	" 15.40, " 6.51

#### DISCUSSION

The aldehydic properties of *l*-mannuronolactone were demonstrated by the following reactions: reduction of Fehling's solution when warmed with that reagent, positive reaction with naphthore-

<sup>1</sup> The *d* component obtained either by synthesis or from natural sources melts at 142–143° and has  $[\alpha]_D^{25} = +92^\circ$  (11–13).

sorcinol and hydrochloric acid, and reduction of ammoniacal silver nitrate at room temperature. The brucine salt also yielded positive reactions with the above reagents. The above reactions in conjunction with the isolation of the barium *p*-bromophenylhydrazone-*l*-mannuronate establish the validity of the synthesis of *l*-mannuronic acid.

In determining the melting point of a mixture of *l*-mannuronolactone and *d*-mannuronolactone (the latter either synthetic (11) or isolated from natural sources (13)) it was observed that the mixture sintered at 138–140° and then melted with decomposition above 155°. Since the individual *d* and *l* components melt at 142–143°, it is probable that the sintering observed at 140° in the determination of the melting point of the mixture represents a transformation into a racemic form having a decidedly higher melting point than either the *d* or *l* component.

The nature of the crystalline substance obtained as a by-product in the course of the isolation of *l*-mannuronolactone from the crude barium salt has not been determined with certainty.

Its melting point agrees with that of  $\delta$ -*l*-mannonolactone, yet the rotation of the compound which is practically 0 eliminates any possibility of this identity. The substance does not reduce Fehling's solution and combines slowly with sodium hydroxide at room temperature or below, indicating the probable presence of a lactone linkage. A study of this compound is in progress.

#### SUMMARY

Crystalline *l*-mannuronolactone has been prepared by the partial reduction of *l*-mannosaccharic acid dilactone.

The *l* compound thus obtained was found to possess the properties predicted by those of its previously known enantiomorph.

We are indebted to our colleague, Dr. Eugene Schoeffel, for conducting the microanalytical determinations.

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# SYNTHESIS OF THE HEXURONIC ACIDS\*

## VI. THE SYNTHESIS OF *L*-GALACTURONIC ACID FROM *L*-GALACTOSE

By CARL NIEMANN AND KARL PAUL LINK

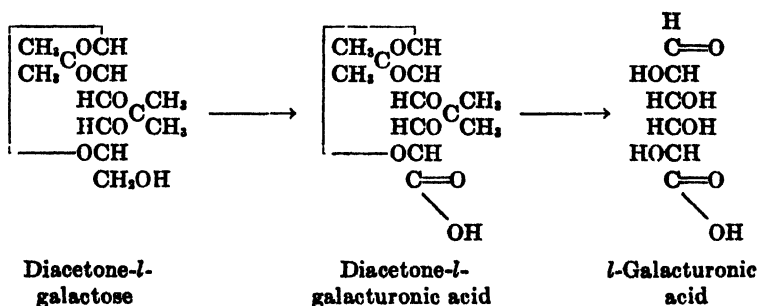
(From the Biochemistry Research Laboratory, Department of Agricultural Chemistry, University of Wisconsin, Madison)

(Received for publication, January 20, 1934)

The synthesis of *L*-galacturonic acid has not been realized in spite of statements in several treatises (1-3) that it has been accomplished. In 1931 we pointed out (4) that the reported synthesis of *L*-galacturonic acid by Kiliani was rescinded by the author himself but unfortunately this observation has been overlooked in the above treatises.

*D*-Galacturonic acid has been synthesized by the oxidation of diacetone-*D*-galactose to diacetone-*D*-galacturonic acid followed by a hydrolysis to the free acid (5-8). Since *L*-galactose is obtainable from flaxseed mucilage (9), we have utilized this material as a starting point for the analogous synthesis of *L*-galacturonic acid. A syrup containing *L*-galactose and *D*-xylose was acetonated and then oxidized with potassium permanganate. The diacetone-*L*-galacturonic acid isolated from the reaction mixture was converted in good yields into *L*-galacturonic acid monohydrate by hydrolysis in an aqueous solution.

The synthesis can be illustrated by the following structural formulæ.



\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by grants from the University Research Fund.

## EXPERIMENTAL

The melting points, rotations, and analyses reported below were conducted under conditions previously described in this *Journal* (10).

*Preparation of a Crude Diacetone-l-Galactose*—An aqueous solution of *l*-galactose and *d*-xylose, isolated from flaxseed mucilage by the method of Anderson (9), was adsorbed on kieselguhr, dried, and then acetonated, the conditions described by Levene and Meyer being used (11).

The product when redistilled under 0.018 mm. pressure at 140–150° was a light yellow viscous oil with an  $[\alpha]_D^{25}$  of +34.0°. An equimolar mixture of diacetone-*l*-galactose and diacetone-*d*-xylose would have an  $[\alpha]_D^{25}$  of +34.5°.

*Preparation of Potassium Diacetone-l-Galacturonate*—13.0 gm. of finely powdered potassium permanganate were added with vigorous stirring to 10.0 gm. of the crude diacetone-*l*-galactose dissolved in 1 liter of 0.6 per cent potassium hydroxide. After 20 hours, the manganese dioxide was removed and the crude potassium salt isolated as previously described for the potassium diacetone-*d*-galacturonate (5). Under these conditions 4.0 gm. of the potassium salt were obtained. For analysis the product was recrystallized from alcohol and ether and dried at 56° under diminished pressure.

*Analysis*—Calculated for  $C_{12}H_{17}O_7K \cdot \frac{1}{2}H_2O$ . K12.40

Found.

" 12.42, 12.44

*Preparation of Diacetone-l-Galacturonic Acid*—3.0 gm. of the potassium salt were dissolved in 7.80 cc. of 1.163 N sulfuric acid and the liberated organic acid extracted from the aqueous solution with six 50 cc. portions of ether. The ethereal extracts were combined and dried overnight with sodium sulfate. Upon spontaneous evaporation of the ether the diacetone-*l*-galacturonic acid crystallized out in fine prismatic needles. The compound was then purified by recrystallization from benzene and petroleum ether and dried at room temperature over phosphorus pentoxide. Yield 1.2 gm.

*Melting Point*—The acid melted at 152–154°.

*Rotation*— $[\alpha]^{25} = +80^\circ \pm 3^\circ$  (in  $CHCl_3$ ,  $c = 0.9$  per cent).



*Analysis*—Calculated for  $C_{12}H_{18}O_7$ . N. E.\* 36.48 cc. 0.1 N alkali  
 Found. " 37.21 " 0.1 " "

\* N. E. represents the neutralization equivalent.

*Preparation of l-Galacturonic Acid*—0.85 gm. of diacetone-*l*-galacturonic acid was dissolved in 30 cc. of water and the solution heated in a boiling water bath for 3 hours (8). Upon evaporation of the water under reduced pressure the syrup was taken up in 95 per cent ethyl alcohol, filtered, and placed in a desiccator. In 4 to 5 hours the *l*-galacturonic acid crystals formed a solid cake. After triturating the mass with 95 per cent ethyl alcohol, the crystalline acid was removed from the mother liquors and dried over phosphorus pentoxide for several days. Yield 0.4 gm.

*Melting Point*—The monohydrate sintered at 112–113° and melted with decomposition at 162–163°.

*Rotation*— $[\alpha]_D^{21} = -56^\circ \pm 3^\circ$  (in water,  $c = 1.08$  per cent).

*Analysis*

Calculated for  $C_6H_{10}O_7 \cdot H_2O$ . N. E. 47.20 cc. 0.1 N alkali, C 33.96, H 5.66  
 Found. " 47.18 " 0.1 " " " 34.10, 34.13,  
 H 5.63, 5.71

*Preparation of the Oximehydroxylamine Salt of l-Galacturonic Acid*<sup>1</sup>—The mother liquor from the *l*-galacturonic acid crystallization was taken up in 10 cc. of 95 per cent ethyl alcohol and heated to boiling on the steam bath. 10 cc. of 1 per cent alcoholic hydroxylamine acetate solution were then added and the reaction mixture cooled. The crude oximehydroxylamine salt was recovered, recrystallized from an aqueous alcohol mixture, and dried at room temperature over phosphorus pentoxide. Yield 0.10 gm.

*Melting Point*—The compound melted at 151–152° with decomposition.

*Analysis*—Calculated for  $C_6H_{14}O_5N_2 \cdot H_2O$ . N 10.77  
 Found. " 10.40, 10.42

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<sup>1</sup> The oximehydroxylamine salt of *d*-galacturonic acid had been prepared by us (unpublished data) by adding 1 equivalent of *d*-galacturonic acid in an alcoholic solution to a boiling alcoholic solution containing 2 equivalents of hydroxylamine acetate. The compound recrystallized from aqueous alcohol; e.g., the monohydrate (N = 10.62 per cent) melted at 151–152° with decomposition.

## DISCUSSION

*l*-Galacturonic acid, as expected, exhibits all of the chemical and physical properties that are characteristic of its *d*-enantiomorph.

In a previous communication we have described several phenyl- and *p*-bromophenylhydrazine derivatives of *d*-galacturonic acid that are useful for the characterization of this acid (10). In continuing our investigations of characteristic derivatives of the uronic acids we have found that the oximehydroxylamine salts of the uronic acids are well defined and easily purified compounds.

Therefore, for the future characterization of either *d*- or *l*-galacturonic acid, we suggest that the preparation of the oximehydroxylamine salt should be seriously considered.

## SUMMARY

Crystalline *l*-galacturonic acid has been synthesized by the oxidation of diacetone-*l*-galactose to diacetone-*l*-galacturonic acid, which in turn was converted into the crystalline free acid by hydrolysis in aqueous solution.

The preparation of the oximehydroxylamine salt of both *d*- and *l*-galacturonic acids has been described.

We are indebted to Dr. Eugene Schoeffel for conducting the microanalytical determinations and to Mr. James Sprague for his aid in the preparation of the oximehydroxylamine salt.

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## THE PHOSPHATASE HYDROLYSIS OF DIPHOSPHO-*L*-GLYCERIC ACID

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(Received for publication, January 16, 1934)

In the attempt to estimate the distribution of the phosphorus compounds in blood, it is obviously of importance to parallel the procedures involved on such compounds as have already been isolated. The indications in the literature and the statement by Robison (1) that diphospho-*L*-glyceric acid constitutes a large if not the entire portion of the fraction of acid-soluble ester not readily hydrolyzed by bone phosphatase warrant a study of the conditions determining the enzymic hydrolysis of that compound.

Diphospho-*L*-glyceric acid was isolated from the blood by Greenwald (2) in 1925. He obtained from 12.2 to 19.3 mg. per cent as phosphorus from pig blood or about 36 to 55 per cent of the organic acid-soluble phosphorus. Similarly, he obtained yields which amounted to about 25 per cent of the organic acid-soluble fraction from dog and human blood, but none from beef blood. Theoretical estimates of the diphospho-*L*-glyceric acid fraction have been higher. According to Jost (3), who calculated this fraction from the lead-precipitable phosphorus of a protein-free filtrate, 75 to 80 per cent of the organic acid-soluble phosphorus of human whole blood is in this form. Kay and Robison (4) obtained in man and in a variety of animals a bone phosphatase-hydrolyzable fraction of 14 to 36 per cent; according to Robison, therefore, the diphospho-*L*-glyceric acid might amount to 64 to 86 per cent of the organic acid-soluble phosphorus.

Jost found that diphospho-*L*-glyceric acid was hydrolyzed to a considerable extent by kidney phosphatase during 3 hours at 42° in a 2 per cent sodium bicarbonate solution; under the same condi-

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tions the action of bone, spleen, and muscle extracts was very slight. Roche (5) found little action by bone, intestine, or kidney extracts; he considered pH 8.8 as optimal for the bone phosphatase action, and 8.6 as optimal for the kidney and intestinal extracts. Kobayashi (6) found that the rate of hydrolysis of diphospho-*l*-glyceric acid by taka-phosphatase (*Aspergillus oryzae*) was maximal at two pH levels, 2.9 and 5.5, and, in the region between these points, minimal at 4.0. His report also contains data showing a greater rate of hydrolysis of diphospho-*l*-glyceric acid by rabbit bone and pig kidney phosphatases at pH 8.0 than at higher pH levels.

TABLE I  
*Hydrolysis of Diphospho-l-Glyceric Acid by Phosphatases from Various Sources*

Concentration of diphospho-*l*-glyceric acid as phosphorus, 9.35 mg. per 100 cc. of hydrolysis mixture; of enzyme extract, 33.3 per cent; of sodium diethylbarbiturate buffer, 0.27 per cent; pH about 9.0 (colorimetric).

Enzyme preparation	Mg. P liberated as inorganic phosphate per 100 cc. hydrolysis mixture in					
	1 day	3 days	7 days	10 days	16 days	19 days
Rat bone (Preparation RBD)...	1 45	4 55	7 65	8 77	9 27	9 37
Cattle bone (Preparation CBB)...	1 25	4 13	7 30	8 25	9 25	9 40
Rat kidney ( " RKD).	2 03	6 08	8.58	9 33	8 83	9 73
" intestine (Preparation RIC).	8 02	9 47	9 92	10 02	9 47	9 32
Cattle intestine (Preparation CIA).....	8.95	9.10	9.45	9.70	9.55	9 70

*Rate of Hydrolysis of Diphospho-l-Glyceric Acid by Phosphatase*—The enzymes were obtained from various tissues by aqueous extraction. The method of preparing the crude extracts, and the technique of determining activity have been described elsewhere (7). Hydrolysis was carried out at room temperature (22–25°) unless otherwise stated. The diphospho-*l*-glyceric acid was prepared as the penta- or tribarium salt according to Greenwald. It was dissolved in water with the addition of a minimal amount of hydrochloric acid; the barium was precipitated by means of a few drops of concentrated sodium sulfate and the solution filtered and made up to a definite volume.

Diphospho-*l*-glyceric acid is completely hydrolyzed by bone and intestinal extracts of rat and cattle and by kidney extracts of rat at a pH of about 9.0 (Table I). The intestinal phosphatase acts

TABLE II

*Effect of pH upon Rate of Hydrolysis of Diphospho-*l*-Glyceric Acid by Phosphatase*

Enzyme preparation	Enzyme concentration	Concentration of diphospho- l-glyceric acid per 100 cc. hydrolysis mixture, as P	Reaction velocity ( $Q_{0.02} \times 10^4$ ) at pH									
			6.8	7.2	7.4	7.6	7.8	8.0	8.2	8.4	8.6	9.2
Bone phosphatase (22-25°)												
Cattle bone (CBD); 0.32 mg. Mg per cc. en- zyme	12.5	3.5	0.9	1.2	1.3	1.2	1.1	0.9	0.7			
Cattle bone (CBD)	37.5	18.1	3.2	5.6	6.8	7.4	7.5	7.1	5.8	3.6		
Rat bone (RBE)	12.5	9.1			1.5	1.5	1.4	1.2	1.0	0.8	0.4	0.0
Intestinal phosphatase (24.0°)												
Rat inter- stine (RIE)	12.5	5.0				42	44	47	50	52	54	30
Cattle in- testine (CIA)	37.5	9.1			80	100	120	142	158	158	135	60

\*  $Q_{0.02}$  is the reciprocal of the time in minutes necessary to liberate 0.02 mg. of phosphorus as inorganic phosphate per cc. of hydrolysis mixture. The concentration of diethylbarbiturate buffer is 0.5 per cent; pH determined colorimetrically.

much more rapidly than the kidney or bone; whereas about 90 per cent of the compound in the hydrolyzing mixture containing

## 750      Dipospho-*L*-Glyceric Acid Hydrolysis

9.35 mg. as phosphorus per 100 cc. was hydrolyzed by the intestinal extract in 1 day, about 10 days were required by the bone and

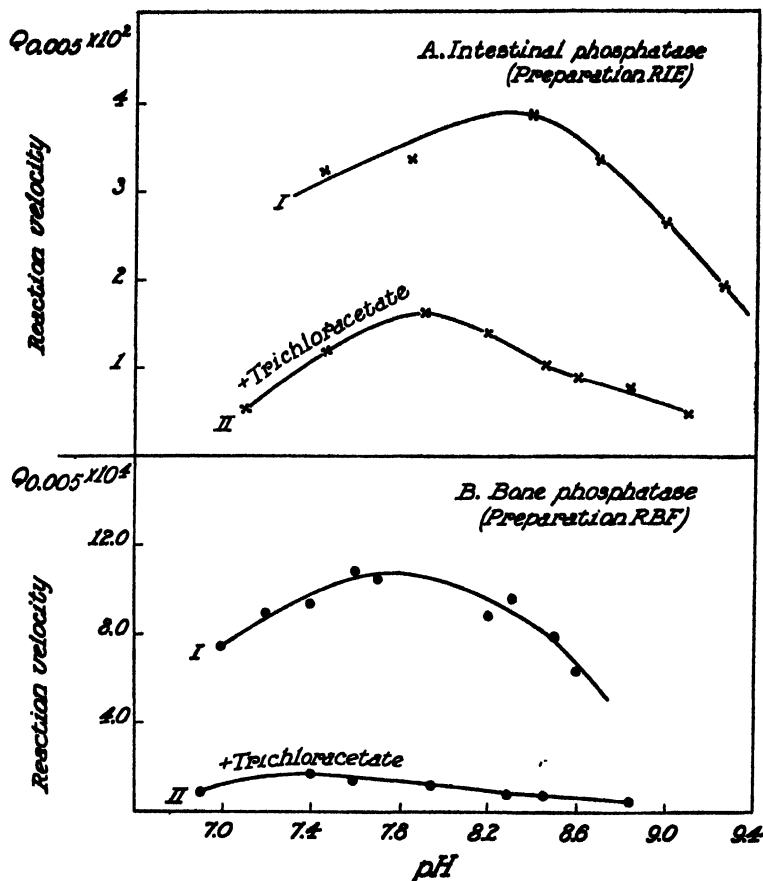


FIG. 1, A AND B. Effect of 4 per cent trichloroacetate on the hydrolysis of dipospho-*L*-glyceric acid by phosphatase. Concentration of enzyme, 12.5 per cent; concentration of dipospho-*L*-glyceric acid as phosphorus, 3.0 mg. per 100 cc. of hydrolysis mixture in B, 9.0 mg. per 100 cc. of hydrolysis mixture in A; diethylbarbiturate buffer; 25° in A, room temperature in B. Curve I, no trichloroacetate; Curve II, 4 per cent trichloroacetate, calculated as acid. pH determinations, colorimetric.

kidney extracts. The much more rapid action of intestinal phosphatase is also illustrated in Table II. The rat bone phosphatase,

Preparation RBE, shows an activity 0.3 that of the intestinal phosphatase of cattle, Preparation CIA, when sodium glycerophosphate is used as substrate. When diphospho-*l*-glyceric acid is employed as substrate, the activity of Preparation RBE is about 0.03 that of Preparation CIA.

The whole blood phosphatase of hemolyzed blood also effects complete hydrolysis. Diphospho-*l*-glyceric acid was added to two hemolyzed bloods so as to correspond to a concentration as phos-

TABLE III

*Retardant Effect of Trichloroacetate on Hydrolysis of Sodium Glycerophosphate by Bone Phosphatase*

0.0127 M sodium glycerophosphate; 0.5 per cent sodium diethylbarbiturate buffer.

Enzyme preparation	Temperature	Enzyme concentration	Concentration of trichloroacetate, as trichloroacetic acid, in hydrolysis mixture	$Q_{0.05}$ * Reaction velocity	Retardation
	°C.	per cent	per cent		per cent
Cattle bone (CBD)	24	25	0	0.0143	
			4.7	0.0063	56
Cattle bone (CBD); 0.19 mg. Mg per cc. enzyme	24	50	0	0.0340	
			2.5	0.0227	33
Rat intestine (RIE)	27	12.5	0	0.0135	
			2	0.0126	7
			4	0.0135	0
	30	25	0	0.0223	
			4	0.0217	3

\*  $Q_{0.05}$  is the reciprocal of time in minutes necessary to liberate 0.05 mg. of phosphorus as inorganic phosphate per cc. of hydrolysis mixture.

phorus of 18.5 mg. per 100 cc. of blood. About 70 to 80 per cent hydrolysis of the added compound occurred within 7 days, and hydrolysis was complete in 20 days.

Control solutions containing no enzyme and adjusted to a pH of about 9.0 showed no hydrolysis of diphospho-*l*-glyceric acid during a period of 15 days. Similarly, the inorganic phosphate content of solutions containing enzyme but no substrate remained constant.

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**pH Optimum**—The relation between pH and phosphatase hydrolysis of diphospho-*l*-glyceric acid is shown for three bone and two intestinal preparations in Table II; additional curves are shown in Fig. 1. The optimal pH for the action of bone phosphatase lies at about 7.4 to 7.8, for intestinal phosphatase at about 8.2 to 8.6. These are distinctly different from the optima, about

TABLE IV

*Influence of Concentration of Trichloroacetate on Hydrolysis of Diphospho-*l*-Glyceric Acid by Bone Phosphatase at pH 8.7 to 8.8*

25 per cent dialyzed cattle bone phosphatase; concentration of diphospho-*l*-glyceric acid as phosphorus, 1.41 mg. per 100 cc. of hydrolysis mixture; sodium diethylbarbiturate buffer; room temperature. The zero value is calculated from mixtures containing similar proportions of components in trichloroacetic acid. Duplicate hydrolyses were carried out.

Concentration of trichloroacetate calculated as trichloroacetic acid  per cent	Mg P liberated per 100 cc. hydrolysis mixture in						
	0 days	6 days	14 days	21 days	31 days	50 days	∞
0	0 25	1.17	1 46	1.76	1.75	1.58	1.66
		1.09	1.45	1.57	1.59	1.54	
1	0.25	0.60	0.79	0.87	1.04	1.06	1.66
		0.53	0.83	0.93	1.06	1.09	
2	0 25	0.45	0.57	0.62	0.59	0.64	1.66
		0.42	0.52	0.57	0.59	0.60	
3	0.25	0 37	0.41	0.53	0.43	0 44	1.66
		0 37	0.39	0.45	0.42	0.43	
4	0.25	0.37*	0.40	0.41	0.39	0.38	1.66
		0.34	0.41	0 39	0.35	0.36	

\* This small difference between the zero and first readings did not appear in such experiments when phosphate was added to the sample so as to read against a stronger standard.

pH 9.0, for the hydrolysis of sodium glycerophosphate. In the more alkaline regions, the hydrolysis rate of diphospho-*l*-glyceric acid by bone phosphatase decreases rapidly, being about one-half the optimal at pH 8.4. At pH 9.2 no liberation of inorganic phosphate from diphospho-*l*-glyceric acid occurred during 300 hours, though the enzyme was still active at the conclusion of that period as shown by its hydrolysis of sodium glycerophosphate.



**Retardant Effect of Trichloroacetate on Activity of Phosphatase—**Studies on the phosphatase hydrolysis of the organic acid-soluble phosphorus of blood are most conveniently carried out on the trichloroacetic acid filtrates. Kay and Robison (4) attempted the removal of trichloroacetate by ether extraction before hydrolysis but they abandoned their attempts, stating that the trichloroacetate did not interfere with the bone phosphatase hydrolysis.

TABLE V

*Influence of Concentration of Trichloroacetate on Hydrolysis of Filtrate of Pig Blood by Bone Phosphatase at pH 8.7 to 8.8*

Concentration of filtrate, 12 per cent; concentration of dialyzed cattle bone phosphatase, 18 per cent; concentration of organic acid-soluble phosphorus, 0.90 mg. per 100 cc. of hydrolysis mixture; sodium diethylbarbiturate buffer; room temperature. The zero value is calculated from the inorganic phosphate content of enzyme and blood filtrate. Duplicate hydrolyses were carried out.

Concentration of trichloroacetate calculated as trichloroacetic acid	Mg. P liberated per 100 cc. hydrolysis mixture in							
	0 days	9 days	11 days	18 days	25 days	28 days	38 days	∞
<i>per cent</i>								
0.98	0.31	0.53 0.60	0.58 0.72	0.69 0.87	0.96 1.03	0.98 1.03	1.03 1.08	1.21
1.96	0.31	0.56 0.55	0.58 0.58	0.69 0.64	0.74 0.78	0.74 0.77	0.84 0.86	1.21
2.94	0.31	0.53 0.52	0.52 0.55	0.54 0.53	0.62 0.63	0.62 0.61	0.70 0.69	1.21
3.92	0.31	0.53 0.52	0.54 0.53	0.49 0.50	0.56 0.58	0.62 0.55	0.62 0.62	1.21
4.90	0.31	0.51 0.50	0.50 0.50	0.47 0.47	0.56 0.51	0.54 0.51	0.56 0.54	1.21

Table III shows the strong retardant action which the trichloroacetate ion exerts on the hydrolysis of sodium glycerophosphate by bone phosphatase; in contrast, there is no retardant action on the hydrolysis by intestinal phosphatase. Fig. 1 shows the effect of the trichloroacetate ion on the hydrolysis of diphospho-L-glyceric acid. Thus a concentration of 4 per cent trichloroacetate, calculated as the acid, reduces at optimal pH the activity of the bone

## 754      Diphospho-*l*-Glyceric Acid Hydrolysis

phosphatase,  $Q_{0.005}$  (the reciprocal of the time in minutes necessary to liberate 0.005 mg. of phosphorus as inorganic phosphate per cc. of hydrolysis mixture), from  $9.8 \times 10^{-4}$  to  $1.5 \times 10^{-4}$ . The effect on the hydrolysis by intestinal phosphatase is not quite so marked, the reduction being from  $3.85 \times 10^{-3}$  to  $1.62 \times 10^{-3}$ .

Table IV shows the effect of varying concentrations of trichloroacetate ion on the rate of hydrolysis of diphospho-*l*-glyceric acid by bone phosphatase at pH 8.7 to 8.8. The rate decreases as the concentration rises until at a 4 per cent concentration, *there is no progressive hydrolysis of the diphospho-*l*-glyceric acid during 50 days.*

Table V shows parallel results with the trichloroacetic acid filtrate of pig blood. When this is diluted so that the concentration of the trichloroacetate is 0.98 per cent, the hydrolysis of the organic acid-soluble phosphorus proceeds gradually and progressively until it is almost complete in 40 days. At the higher concentrations of trichloroacetate, a 30 per cent fraction is hydrolyzed within the first few days, but the hydrolysis of the remaining phosphorus compounds proceeds very slowly. At 4 and 5 per cent concentrations of trichloroacetate, the hydrolysis beyond that of the first 30 per cent is practically nil. The phosphatase remains active under these conditions.

### DISCUSSION

The preceding work shows that the rate of hydrolysis of diphospho-*l*-glyceric acid by bone phosphatase is extremely slow, that the optimal pH is in the neighborhood of 7.5, and that the rate is greatly depressed by the presence of trichloroacetate ion. In contrast, the rate of hydrolysis by intestinal phosphatase is much more rapid; the optimal pH is about 8.4, and the retardant effect of trichloroacetate is not so pronounced.

In determining the fraction hydrolyzable by bone phosphatase, Kay and Robison employed a mixture of 10 cc. of bone extract and 100 cc. of blood filtrate which contained 3 to 4 per cent of trichloroacetic acid. They chose a pH of 9.0 to 9.3 which they assumed optimal, and conducted the hydrolyses for 5 hours, finding no further liberation of inorganic phosphate in the following hours. Other investigators have observed similar conditions. As can be seen from our work, Kay and Robison fortuitously chose conditions which would effectively prevent the hydrolysis of any diphospho-*l*-glyceric acid present in the filtrate.

The work presented also explains the finding of King (8) that at a pH of 7.0, 84 per cent of the organic acid-soluble phosphorus of blood is hydrolyzed by bone phosphatase in 2 to 3 days at 37°. Examination of King's experimental procedure shows that the concentration of trichloroacetate in the final hydrolyzing solution was 1.5 per cent. This concentration is much lower than that employed by Kay and Robison and, according to Table V, exerts only a slight retardant effect on the hydrolysis by bone phosphatase. pH 7.0 is near the optimal zone for the hydrolysis of diphospho-*l*-glyceric acid and perhaps, under the conditions of the experiment, within the zone. The favorable pH and the low concentration of trichloroacetate permit the hydrolysis of diphospho-*l*-glyceric acid in the blood filtrates, as employed by King.

#### SUMMARY

The comparative rates of hydrolysis of diphospho-*l*-glyceric acid by bone and intestinal phosphatases, the zone of optimal pH action, and the influence of trichloroacetate ion upon the hydrolysis were studied. Diphospho-*l*-glyceric acid resides entirely in the fraction of the organic acid-soluble phosphorus of blood not hydrolyzable by bone phosphatase at pH 9.0 and 4 per cent trichloroacetate concentration.

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## THE ACID-BASE BALANCE OF THE MINERALS RETAINED DURING HUMAN PREGNANCY\*

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No information seems to be available as to the relative amounts of acid- and base-forming elements actually used by the human organism during normal pregnancy. Acid-base balances have been determined in growing infants and children (1, 2). These growth factors exist during pregnancy, and in addition, the demands of the adult organism, whatever they may be, for material to be used in the preparation for and performance of the special tasks of gestation, parturition, and lactation. Probably the nearest approach to a quantitative estimation of one phase of this complex requirement is that furnished by Shohl (3). Calculating from existing data on the composition of the fetus, he found that an average of 85 cc. of 0.1 N base in excess of acid were deposited daily during the last 100 days of gestation and suggested that the diet of the pregnant woman should provide at least 150 cc. excess of 0.1 N base daily to meet this fetal need. Such fundamental physiologic facts are needed to supplement the numerous studies of the acid-base equilibrium and alkali reserve of the blood during pregnancy and to facilitate an understanding of certain complications of metabolism in abnormal gestation. They would aid in the interpretation of calcium, phosphorus, and magnesium balances, of which too few exist, and in the accurate estimation of the complete nutritional needs of human pregnancy.

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Two major difficulties have hindered attempts to determine the acid-base balance in such subjects. One has been the long tedious analyses necessary for measuring the amounts of acid- and base-forming elements in biological materials. The second and more significant one has been the finding of normal subjects showing satisfactorily positive retentions of calcium, phosphorus, and magnesium and thereby indicating typical metabolism of the other elements involved. By normal subjects is meant healthy women in the course of average uneventful pregnancies with no early nausea to deplete the chlorides and other reserves or cause fasting acidosis, and with few or none of the digestive disturbances common to pregnancy and relieved by the frequent use of bicarbonates or other alkalizing compounds. There should be no toxemias involving upsets in water and electrolyte metabolism, even though not contemporary to the balance experiment itself, and certainly no prematurities to imply abnormal exchange between the maternal organism and the fetus.

In this paper twenty records of acid-base balance during pregnancy on five normal women are presented with the hope that other laboratories will add to the accumulation of data until the evidence is ample to justify definite, tenable conclusions.

#### EXPERIMENTAL

*General Methods*—In this study the women lived at home where they followed their usual routine of activity and eating habits. Aliquot samples of food, drinking water, and urine, and all of the feces for the 4 day metabolism periods were collected, composited, and preserved for analysis. Descriptions of the subjects, the methods of collection, and handling of materials, as well as the proximate composition of the diets, have been reported elsewhere (4).

*Analyses*—Gravimetric procedures were employed for all analyses except those for chlorides. For these the Volhard method was used, following an ashing by the open Carius technique with the modification that the mixture was allowed to stand 12 to 24 hours in the cold and then was digested on a *water* bath 8 to 12 hours. Determinations of chlorides in urine were made on the fresh sample. Total sulfur was determined as  $\text{BaSO}_4$  after slow ashing with  $\text{Cu}(\text{NO}_3)_2$  at a temperature below redness, according to the

method of Benedict and Denis. Phosphorus was precipitated from a solution of the material ashed with a mixture of nitric and sulfuric acids by the Neumann method, first as molybdate, then as magnesium ammonium phosphate, and ignited to the pyrophosphate. Sodium and potassium were obtained as chlorides and the potassium separated as perchlorate. Calcium and magnesium were determined by the modified McCrudden method, methyl red being used to control the pH of the solution in the separation of the two elements. Details and original references for the above methods are to be found in the book by Peters and Van Slyke (5).

All analyses were made in triplicate and repeated when necessary to obtain reproducible results in triplicate. A review of the difficulties encountered in the application of these procedures to different biological materials would be too lengthy to be included in this paper. Results are reported here only to the second decimal figure, although all weighings were made to the fourth decimal place and the computations included the fourth significant figure. The monovalent equivalents used in the calculations of potential acidity and alkalinity were 2 for Ca, Mg, and S, 1.8 for P (1, 5) and 1 for each of the other elements.

### *Results*

Tables I and II show the weights of acid-forming and of base-forming elements, respectively, which were consumed in the food and water and excreted by way of the urine and feces. Table III summarizes the volumes of 0.1 N fixed base and excess base in the intake, urine, feces, and amounts retained, as computed from the weights in Tables I and II. The total volumes of 0.1 N acid can be ascertained from Table III by difference between total and excess base.

A brief discussion of the individual elements will include only those points significant to the acid-base economy. Attention is directed first, therefore, to the number and extent of the base balances in the retentions as shown in Table III. In every instance an excess of the base-forming elements was stored, the amount being greater than the 150 cc. of 0.1 N base daily allowed by Shohl (3) in all but two of the twenty balances. The retention was over three times this allowance in half of the experimental periods. This may be some indication of the magnitude of the maternal needs exclusive of fetus.

**Chlorides**—The metabolism of chlorides showed wide variations. Retentions ranged from  $-0.23$  to  $2.16$  gm. of Cl daily (Table I), with a mean of  $0.89 \pm 0.10$ , but the variations were not related to any period of pregnancy. Most noteworthy was the fact that a negative or low Cl balance was associated frequently with a high P one (Case II, Period 1 and Case VI, Period 1); and *vice versa*, low

TABLE I  
*Acid-Forming Elements Retained by Women during Pregnancy.*  
*Daily Averages in Gm.*

Case No.	Period No.	Wk. of pregnancy	Chlorine				Sulfur				Phosphorus			
			Intake	Urine	Feces	Retention	Intake	Urine	Feces	Retention	Intake	Urine	Feces	Retention
I	2	27	4.00	4.12	0.11	-0.23	0.57	0.46	0.09	0.02	1.43	0.65	0.45	0.33
	3	31	3.73	3.18	0.10	0.45	0.65	0.55	0.08	0.02	1.37	0.75	0.43	0.19
	4	34	4.22	3.33	0.11	0.78	0.59	0.54	0.09	-0.05	1.40	0.70	0.51	0.19
	5	38	3.96	3.25	0.17	0.54	0.61	0.55	0.08	-0.02	1.39	0.68	0.45	0.26
II	1	26	4.85	4.79	0.08	-0.02	0.86	0.63	0.12	0.11	1.99	0.85	0.62	0.52
	2	30	5.45	4.68	0.12	0.65	0.84	0.68	0.12	0.04	2.08	0.80	0.61	0.67
	3	34	5.61	3.74	0.06	1.81	0.84	0.68	0.12	0.04	2.07	1.07	0.67	0.33
IV	1	31	6.57	4.74	0.08	1.75	0.73	0.67	0.11	-0.05	1.56	1.05	0.49	0.02
	2	35	6.30	4.51	0.06	1.73	0.64	0.59	0.10	-0.05	1.28	0.78	0.34	0.16
	3	39	9.68	8.15	0.27	1.25	0.74	0.63	0.12	-0.01	1.61	0.96	0.30	0.35
V	1	28	6.49	5.75	0.04	0.70	0.63	0.58	0.08	-0.03	1.18	0.62	0.34	0.22
	2	33	5.03	4.09	0.07	0.87	0.60	0.51	0.10	-0.01	1.05	0.47	0.43	0.15
	3	38	6.75	4.52	0.07	2.16	0.64	0.54	0.11	-0.01	1.26	0.59	0.43	0.24
	4	39	6.12	5.74	0.06	0.32	0.55	0.46	0.09	0.00	1.27	0.67	0.42	0.18
VI	1	18	8.26	7.22	0.21	0.83	1.36	1.12	0.26	-0.02	2.56	0.96	0.82	0.78
	2	23	6.85	6.01	0.25	0.59	0.94	0.75	0.15	0.04	2.34	1.00	0.98	0.36
	3	27	6.08	4.32	0.20	1.56	0.81	0.70	0.12	-0.01	1.53	0.86	0.56	0.11
	4	31	5.76	4.09	0.33	1.34	0.97	0.64	0.28	0.05	1.83	0.79	0.76	0.26
	5	35	5.13	4.08	0.23	0.82	0.77	0.55	0.15	0.07	1.84	0.80	0.68	0.38
	6	38	5.59	5.62	0.13	-0.16	0.80	0.68	0.16	-0.04	1.75	0.73	0.64	0.38

P storage accompanied high Cl (Case II, Period 3 and Case VI, Period 3). Perhaps this behavior was merely the result of the sparing action of chlorides in maintaining the acid-base equilibrium of body tissues and fluids in the absence or presence of conditions favoring P retention. However, neither an excess of acid-forming nor a large predominance of the base-forming elements in the



urine, to the exclusion of organic acids, seemed related to the variations in Cl retentions (Cases IV and VI; also Table III).

With only one of the three negative balances of Cl was the Na balance also negative (Case VI, Period 6), indicating an elimination of previously retained NaCl. This explanation was supported by the disappearance of a slight edema which had existed at the beginning of the metabolism period. Abnormal sweating did not account for the higher Cl retentions, because most of the periods showing these came during the winter and spring months, while the negative Cl balances occurred in summer or early fall. Also, the same individual during consecutive periods with similar intake did not show comparable differences between intake and outgo for Cl (Case V, Periods 3 and 4). More or less error from losses in perspiration existed, doubtless, in all of the chloride balances.

The Cl intakes were low in these diets, particularly those of Cases I, II, and VI, due to intentional restriction of the amount of table salt used. The intakes ranged from 4.00 to 9.68 with a mean of 5.82 gm. of Cl daily.

*Sulfur*—The large number of negative S balances invites critical inspection. In the first place both negative and positive balances were small, less than 10 per cent of the intake except in one instance (Case II, Period 1). Nine of the twenty balances were equivalent to 3 per cent or less of the intake and were, therefore, within the range of experimental error for an intake-outgo study. The daily requirement of this element for fetal development is probably not large. Assuming that 14.5:1 is the average nitrogen to sulfur ratio for tissue protein (6) and 72.2 gm. the nitrogen content of the mature human fetus (7), then approximately 5 gm. total or 0.02 gm. daily throughout gestation would be required for the soft tissues. Additional quantities would be needed for bony structure and probably for the maternal organism. These facts suggest, however, that more refined methods for balance experiments and for the analyses of biological materials for S content may be necessary in order to study the fluctuations in the metabolism of this element.

In view of the preponderance of base-forming elements in the intakes and excretions (Table III), one might expect that S would be sacrificed to save P of the acid-forming elements. The varia-

tions in the amounts of S retained were too small to permit deductions on this point. The negative balances seemed in no way related to the excess of base in the intake or urine.

Positive retentions for S were not dependent on high total intakes of this element (Cases I and VI), although more storage did occur on the diets containing large proportions of eggs and milk (Cases II and VI) (4, 8). The nutritional availability of the S in diets supplying considerable quantities of fruits and vegetables, particularly in those of Case IV, is questionable. All diets were more or less restricted as to meat proteins (4), but the nitrogen to sulfur ratio varied only from 12 to 20, averaging 15. Also, this ratio in the urine ranged from 11 to 16, averaging 13; whereas ratios of 13 to 16 constitute a normal range for fasting animals in which tissue proteins only were catabolized (6). Hence these diets were not considered grossly deficient in S content. It is important in this connection to point out that nitrogen retentions for the group as a whole were low (8) and that high nitrogen storage tended to be associated with S retentions. It would appear from these facts that the metabolism of S and factors influencing it during pregnancy deserve to be investigated further.

*Phosphorus*—Phosphorus retentions ranged from 0.02 to 0.78, with an average of  $0.30 \pm 0.03$  gm. daily. Thus there were no negative balances but a number were comparatively low (Cases I, IV, and V). Evidence of this is to be found in the Ca:P ratio of the amounts stored. The ratios were well above 1:1 in eleven of the balances, above 2:1 in two instances, and averaged 1:1.2, which is higher than any previously reported for women during pregnancy ((9) and references cited there). A relative excess of Ca in the diet was not the cause of the low P retentions since the Ca:P ratio of the intake was never above 1:1 and averaged only 1:0.88. Relative P deficiency was also evidenced by the fact that the storage in this group was approximately 85 per cent above that for a group reported earlier (9), while the Ca retained in the present series averaged 200 per cent better.

The highest P retentions in this series were coincident with high fixed base retentions for each subject. This observation is supplemented by the record of Case III, omitted from this study because  $\text{NaHCO}_3$  was ingested to relieve gastric distress. The P balances for her were practically in equilibrium due to the excessive excretion of this element incident to a highly alkaline urine.

**Calcium and Magnesium**—No negative balances were found for either Ca or Mg. Table II shows that good storage of these two elements usually was parallel, indicating that some factors which favor retention were common to the two. The data lend no support to the theory that Mg is substituted for Ca in a deficiency of the latter (Cases IV and V). Calcium retention ranged from 0.06

TABLE II  
*Base-Forming Elements Retained by Women during Pregnancy. Daily Averages in Gm.*

Case No.	Period No.	Wk. of pregnancy	Calcium				Magnesium				Sodium				Potassium			
			Intake	Urine	Feces	Retention	Intake	Urine	Feces	Retention	Intake	Urine	Feces	Retention	Intake	Urine	Feces	Retention
I	2	27	1.25	0.08	0.98	0.19	0.33	0.10	0.21	0.02	3.86	2.28	0.24	1.34	3.53	2.89	0.38	0.26
	3	31	1.20	0.06	0.80	0.34	0.36	0.09	0.19	0.08	4.03	2.04	0.28	1.71	3.11	2.66	0.29	0.16
	4	34	1.29	0.06	1.02	0.21	0.29	0.10	0.19	0.00	3.62	2.18	0.29	1.15	3.50	2.83	0.31	0.36
	5	38	1.24	0.08	0.84	0.32	0.32	0.11	0.17	0.04	3.99	2.71	0.29	0.99	3.29	2.46	0.33	0.50
II	1	26	1.80	0.36	1.07	0.37	0.45	0.10	0.24	0.11	4.54	2.94	0.25	1.35	4.47	3.46	0.30	0.71
	2	30	1.77	0.28	1.03	0.46	0.51	0.10	0.25	0.16	4.75	2.94	0.29	1.52	4.34	3.00	0.33	1.01
	3	34	1.82	0.37	1.18	0.27	0.52	0.14	0.34	0.04	5.23	3.14	0.22	1.87	4.35	3.56	0.31	0.48
IV	1	31	1.02	0.59	0.37	0.06	0.45	0.12	0.30	0.03	5.10	3.79	0.21	1.10	3.56	2.49	0.62	0.45
	2	35	1.24	0.56	0.46	0.22	0.39	0.13	0.23	0.03	5.43	3.62	0.26	1.55	4.10	3.02	0.43	0.65
V	1	39	1.56	0.54	0.53	0.49	0.44	0.12	0.21	0.10	7.27	4.93	0.56	1.78	4.41	3.21	0.52	0.68
	2	28	0.89	0.16	0.41	0.32	0.33	0.08	0.16	0.09	5.36	4.28	0.08	1.00	3.16	2.54	0.23	0.39
	3	33	0.81	0.16	0.52	0.13	0.29	0.06	0.22	0.01	3.98	3.30	0.13	0.55	2.30	1.66	0.39	0.25
	4	38	1.03	0.10	0.61	0.32	0.38	0.06	0.25	0.07	5.08	3.10	0.17	1.81	3.37	2.17	0.44	0.76
VI	1	39	1.07	0.10	0.59	0.38	0.35	0.08	0.22	0.05	4.61	3.80	0.17	0.64	3.17	2.17	0.37	0.63
	2	18	2.37	0.29	1.97	0.11	0.47	0.10	0.32	0.05	7.58	5.49	0.43	1.66	3.93	2.95	0.64	0.34
	3	23	2.08	0.21	1.52	0.35	0.46	0.09	0.30	0.07	5.28	4.01	0.32	0.95	4.25	3.26	0.56	0.43
	4	27	1.45	0.27	1.03	0.15	0.33	0.09	0.18	0.06	4.79	2.99	0.29	1.51	2.91	2.31	0.44	0.16
	5	31	1.75	0.20	1.33	0.22	0.40	0.07	0.28	0.05	5.05	2.68	0.58	1.79	3.87	2.53	0.71	0.63
	6	35	1.86	0.19	1.21	0.46	0.41	0.10	0.23	0.08	4.27	2.63	0.47	1.17	3.71	2.30	0.48	0.93
		38	1.56	0.13	1.11	0.32	0.41	0.05	0.29	0.07	4.31	4.10	0.34	-0.13	3.37	2.49	0.40	0.48

to 0.49 gm. daily with a mean of  $0.28 \pm 0.02$ . Magnesium storage ranged from 0 to 0.16 gm. daily with an average of  $0.06 \pm 0.005$ . The storages for both elements tend to run higher than any hitherto published ((9) and citations) and are well above the average daily deposit of these two elements in the fetus as computed from data recently published by Givens and Macy (10). The highest re-

ported quantity of Ca present in a fetus at maturity was 33 gm., representing a deposit of 0.22 gm. daily for the last half of gestation, if it be assumed, as average values show, that approximately 95 per cent of the total was laid down during this period. Likewise fetal increments based on average composition figures for the same period may be shown to be only 0.15 gm. daily, or 0.21 gm. daily for the last 3 months of gestation. In this series the Ca balances obtained during the last half of pregnancy averaged 0.29 gm. daily, those during the last 3 months, 0.30 gm. daily.

The best Ca retention in this group occurred in the case of diets affording a potential alkalinity of more than 1000 cc. of 0.1 N fixed base daily (Case II, Period 2; Case IV, Period 3; Case VI, Period 5), the poorest retentions in the case of those containing 800 cc. or less of 0.1 N excess base (Case IV, Period 1; Case V, Period 2; Case VI, Period 3). However, not all diets supplying a potential alkalinity of more than 1000 cc. of 0.1 N base in excess of acid daily gave optimal storage, implying that factors other than the acid-base balance of the diets were important to Ca retention. The ratio of base to acid in the diet bore no relation to the quantities of Ca stored. Good storage was associated with high base retention but the latter was due chiefly to the large amounts of Ca retained, since no correlation existed after the proportion of base contributed by the Ca was subtracted from the total.

*Sodium and Potassium*—Sodium, Table II, like Cl for the acid-forming group, was the largest contributor to the total fixed base in the intake, urine, and amounts stored. There was only one negative balance of this element and that occurred along with Cl in almost equivalent quantities following a condition of slight edema. Except in three of the twenty balances Na retentions were fairly constant, from 1 to 2 gm. daily, but were irregular with reference to any stage of pregnancy. Low retentions appeared at the end of gestation as well as near the beginning. The average was  $1.26 \pm 0.07$  gm. daily. The amounts of K retained were more variable, ranging from 0.16 to 1.0, with a mean of  $0.51 \pm 0.03$  gm. daily.

The ratio of Na:K stored was fairly constant, approximately 2.5, for Cases IV and V, but quite irregular in the other subjects. The ratio in the urine was below 5.3, usually considered normal, in all but five of the periods and was below one in six instances.

The ratios in the urine tended to parallel those in the intake and suggested, therefore, that the increased amount of K in the urine had its origin in food rather than in a condition of acidosis requiring an increased elimination of the alkali. The storage of Na and K was not parallel to that of the alkaline earths, Ca and Mg, except that frequently high retentions of K and Ca were coincident (Cases II, IV, and VI).

From Tables I and II it is evident that the frequently low Cl retentions, the often negative S retentions, and the relatively low P storage have resulted in a low total of acid-forming elements retained. In contrast, the base-forming elements, Na, K, Ca, and Mg, showed only one negative balance and a few surprisingly high positive balances, thereby accenting the magnitude of the positive base balances. With the exception of S, the amounts retained were above the estimated fetal requirement and may serve to indicate somewhat the quantities needed in phases of the metabolism of gestation other than the maternal-fetal exchange.

*Acid-Base Equilibrium*—Table III shows that all diets supplied an excess of base-forming elements varying from 561 to 1422 cc. of 0.1 N base daily, with a mean of  $1037 \pm 36$  cc. These values are relatively not so different since the ratios of base to acid in the intake spread over the narrow range from 1.2 to 1.5, with a mean of 1.3. The range for the stools was wider and higher, 1.7 to 2.5, mean 2.1, but were unrelated to the variations in the ratios of the diet. Differences in digestibility and slight changes in the paths of excretion for Ca and Mg could easily explain this wider range in the feces.

As mentioned above, base-forming elements always predominated in the amounts stored but, unlike the excess in the intake, showed considerable variation from period to period and from individual to individual, without any relation to the stage of pregnancy. The storage, as expressed in volumes of 0.1 N excess base daily, ranged from 59 to 770 cc. with a mean of  $436 \pm 28$  cc. The ratios of base to acid ranged from 1.2 to 5.8 and were apparently not dependent upon the intake. The storage ratios were above 2 usually in those balances which were augmented by good Ca retention.

The relative quantities of acid and base in the urine were more variable than in food or feces. In four instances acid-forming

elements actually predominated over the 4 day period, and the excess was low or negligible in nine others. This variation was to be expected from the rôle of the urine in the elimination of electrolytes and in maintaining the acid-base equilibrium of the body tissues and fluids. Changes in factors contributing to the acid-base economy were reflected in the urine, as shown by the number

TABLE III

*Total Base Retained by Women during Pregnancy. Daily Averages in Terms of 0.1 N Alkali*

Case No.	Period No.	Wk. of pregnancy	Intake		Urine		Feces		Retention	
			Total base	Excess base	Total base	Excess base	Total base	Excess base	Total base	Excess base
			cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
I	2	27	3475	1162	1851	21	857	505	767	636
	3	31	3442	1182	1672	-5	751	417	1019	770
	4	34	3356	985	1783	89	876	490	697	406
	5	38	3453	1153	1938	282	769	403	746	468
II	1	26	4379	1322	2426	196	919	464	1034	662
	2	30	4474	1204	2270	61	935	474	1269	669
	3	34	4723	1422	2571	468	1045	558	1107	393
IV	1	31	4004	791	2682	319	675	303	647	169
	2	35	4345	1415	2729	632	639	362	977	421
	3	39	5425	1301	3349	102	818	491	1258	708
V	1	28	3855	948	2655	314	432	175	768	459
	2	33	2960	561	1991	243	593	259	376	59
	3	38	3901	869	2001	49	699	362	1201	458
	4	39	3638	832	2324	27	647	327	667	478
VI	1	18	5873	1217	3372	77	1598	899	903	241
	2	23	4806	925	2755	11	1286	558	765	356
	3	27	3819	713	2099	-54	905	453	815	314
	4	31	4256	968	1973	-41	1331	612	952	397
	5	35	4067	1069	1908	-51	1119	577	1040	543
	6	38	3851	760	2529	101	1045	541	277	118

of negative base balances, by the absence of a fairly constant ratio of base to acid in the urine, and by the lack of correlation with the acid-base proportions in the intake. In other words, the urine, by regulating losses of electrolytes, served to stabilize the retention against the variations of diet, digestion, and metabolic demand.

The question of organic acids in the urine arises in this connec-

tion. The preponderance of fixed base in certain urines was not paralleled by the findings for titratable acidity, free ammonia, and pH estimations (unpublished data). For example, the diets chosen by Case IV were among the most potentially alkaline of any in the group, as predicted by the large amount of fruits and spring vegetables consumed (4, 8) and as confirmed by the analytical data reported in Table III. The urine contained a considerable excess of base-forming elements, but showed the highest titratable acidity, free ammonia, and hydrogen ion concentration of any in the series. It is regrettable that the organic acids in these urines were not titrated. The above facts suggest, however, that appreciable quantities of such acids were being excreted, coming either from fruits and vegetables or from some internal production peculiar to the state of pregnancy. They emphasize the need for more studies on the metabolism of various organic acids found in food materials and the fate of these under different physiological conditions. They indicate that many foodstuffs may need to be evaluated individually as regards their potential basicity in the human organism ((11, 12) and citations). Obviously such information is essential to accurate interpretations of acid-base balances.

#### SUMMARY

Twenty acid-base balances during pregnancy on five normal women show that a preponderance of the base-forming elements was retained in every instance and usually in amounts exceeding the estimated fetal requirement. In terms of 0.1 N acid and base the mean daily retentions with probable error were: acid-forming elements,  $427 \pm 27$  cc.; base-forming elements,  $859 \pm 39$  cc.; and excess of base,  $436 \pm 28$  cc. All diets were potentially alkaline, providing 561 to 1422 cc., mean,  $1037 \pm 36$  cc. of 0.1 N excess base daily.

Fair or good retentions were found for every element except sulfur. Of the 140 intake-outgo balances, all were positive except one Na, three Cl, and eleven S. One S and one Mg balance amounted to equilibrium. The mean daily retentions expressed as gm. for the various elements were as follows: Ca 0.28, Mg 0.06, Na 1.26, K 0.51, Cl 0.89, S 0, and P 0.30.

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## OXIDATION OF AMINO ACIDS BY SILVER OXIDE\*

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In the course of the preparation of dimethylglycine from its hydrochloride, it was observed (1) that addition of excess of silver oxide to a cold aqueous solution of the latter caused the evolution of carbon dioxide, accompanied by the odors of dimethylamine and formaldehyde. Repetition of this experiment at the boiling temperature showed that the following reaction took place with essentially quantitative yields.



As this reaction, if general, might offer certain analogies to the biochemical oxidation of amino acids, its study was extended to other compounds.

The formation of metallic silver on heating an aqueous solution of the silver salt of glycine was observed by Kraut and Hartmann (2) and confirmed by Heintz (3), but no attempt appears to have been made to study the reaction nor to ascertain whether it occurs with other amino acids. Strecker (4) found that alanine yields carbon dioxide, ammonia, and acetaldehyde on boiling with a suspension of lead peroxide. Silver oxide was shown by Nef (5) to cause the oxidation of glycolic acid, but under the conditions adopted the reaction halted with the production of oxalic, formic, and carbonic acids.

The results of a series of qualitative tests are shown in Table I; the quantitative experiments are described in the latter part of this report. Glycine and sarcosine are oxidized in boiling aqueous

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solution by silver oxide in much the same way as dimethylglycine, the former considerably more slowly.



TABLE I

*Behavior of Various Amino Acids and Derivatives with Silver Oxide*

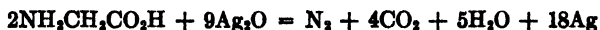
Amino acid	Products recognized
Very rapidly oxidized	
Sarcosine	$\text{CO}_2$ , $\text{CH}_3\text{NH}_2$
Dimethylglycine	" $(\text{CH}_3)_2\text{NH}$
N-Methylalanine	" $\text{CH}_3\text{NH}_2$ , $\text{CH}_3\text{CHO}$ , $\text{CH}_3\text{COOH}$
N-Dimethylalanine	" $(\text{CH}_3)_2\text{NH}$ , $\text{CH}_3\text{CHO}$ , $\text{CH}_3\text{COOH}$
$\alpha$ -Dimethylaminoisobutyric acid	$\text{CO}_2$ , $(\text{CH}_3)_2\text{NH}$ , acetone
Rapidly oxidized	
Glycine	$\text{CO}_2$ , $\text{NH}_3$ , urea (trace)
Alanine	" " $\text{CH}_3\text{CHO}$ , $\text{CH}_3\text{COOH}$ , urea (trace)
$\alpha$ -Aminoisobutyric acid	$\text{CO}_2$ , $\text{NH}_3$ , acetone
Leucine	" " isovaleraldehyde and acid
$\alpha$ -Aminophenylacetic acid	$\text{CO}_2$ , $\text{NH}_3$ , $\text{C}_6\text{H}_5\text{CHO}$ , $\text{C}_6\text{H}_5\text{COOH}$
Phenylalanine	" " $\text{PhCHO}$ , $\text{PhCOOH}$ , $\text{PhCH}_2\text{CHO}$ , $\text{PhCH}_2\text{COOH}$
Glutamic acid	$\text{CO}_2$ , $\text{NH}_3$ , succinic acid
Proline	" volatile base with pyrrole odor
N-Phenylglycine	" $\text{PhNH}_2$ , $\text{PhN:NPh}$
N-Phenylalanine	" " " $\text{CH}_3\text{COOH}$
N-Phenylaminoisobutyric acid	" " " acetone
$\alpha$ -Phenyl- $\alpha$ -aminobutyric acid	" $\text{NH}_3$ , $\text{PhCOEt}$
Acetylglutamine	"
Hippuric acid	" $\text{PhCONH}_2$
$\alpha$ -Benzoylamino-phenylacetic acid	" $\text{PhCHO}$
N-Benzenesulfonylglycine	" $\text{PhSO}_2\text{NH}_2$
$\alpha$ -Ureidopropionic acid	" $\text{NH}_3$ , urea, $\text{CH}_3\text{CHO}$ , $\text{CH}_3\text{COOH}$
$\alpha$ -Phenylureidopropionic acid	$\text{PhNH}_2$
5-Methylhydantoin	$\text{CO}_2$ , $\text{NH}_3$ , urea, $\text{CH}_3\text{COOH}$ , $\text{CH}_3\text{CONHCONH}_2$
3-Phenyl-5-methylhydantoin	$\text{PhNH}_2$
Creatine	

TABLE I—*Concluded*

Amino acid	Products recognised
Slowly oxidized	
Glycylalanine Alanylglycine Alanylalanine <i>p</i> -Toluenesulfonylalanine $\alpha$ -Benzenesulfaminoisobutyric acid Benzenesulfonylleucine $\alpha$ -Benzenesulfaminophenylacetic acid Benzenesulfonylphenylalanine	CO <sub>2</sub> , NH <sub>3</sub> , CH <sub>3</sub> COOH  Isovaleric acid (odor) PhCHO (odor) PhCH <sub>2</sub> CHO (odor)
Not oxidized	
Betaine $\beta$ -Aminopropionic acid Benzoylalanine <i>d</i> -Phthaliminopropionic acid $\alpha$ -Benzoylaminoisobutyric acid Benzoylphenylalanine 5,5-Dimethylhydantoin 3-Phenyl-5,5-dimethylhydantoin	

Betaine, on the other hand, shows no tendency to reduce silver oxide.

An unexpected difference between glycine and its methyl derivatives is the oxidation of a notable proportion (over 10 per cent) of the primary amino group to nitrogen, with the formation of a correspondingly increased amount of metallic silver.

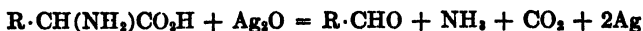


Small but appreciable quantities of urea are also produced. *N*-Phenylglycine, in contrast to sarcosine, shows a similar behavior, yielding azobenzene as well as the anticipated aniline. This fact has, however, no particular significance, since azobenzene is also formed by the action of silver oxide upon aniline itself.

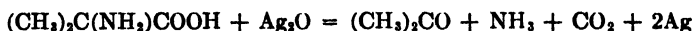
Analogous oxidations occur with homologues of glycine which contain the amino group in the  $\alpha$  position.



That the reaction takes place in at least two stages is shown by the formation of aldehydes

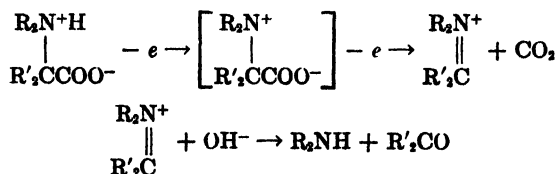


which undergo further oxidation to carboxylic acids, to an extent depending upon experimental conditions and the volatility and solubility of the aldehyde. As with glycine, some oxidation of the amino group to nitrogen takes place with alanine and those of its homologues which contain the grouping  $-CH(NH_2)COOH$ ; this, however, is not the case with N-methylated alanine nor with  $\alpha$ -aminoisobutyric acid (in which no hydrogen atom is present in the  $\alpha$  position). In this last instance the oxidation proceeds only through the first stage, yielding acetone.



That the amino group must be situated in the  $\alpha$  position for oxidation to occur is indicated by the total absence of reaction in the case of  $\beta$ -aminopropionic acid.

Oxidation is suppressed by the presence of either mineral acid or alkali. It thus seems reasonable to suppose that the primary reaction in the case of glycine is a function of the amphion  $^+NH_3 \cdot CH_2 \cdot COO^-$ . Both of the hydrogen atoms of the methylene group, and two of those attached to the nitrogen atom, may be replaced by alkyl groups without adverse influence on the reducing power towards silver oxide. Methylation of the amino group, in fact, leads to increased reactivity, possibly owing to a greater tendency for ionic dissociation in the basic group. Replacement of all three of the hydrogen atoms on the nitrogen, as in betaine, completely inhibits the reaction, which may be expressed in general terms by the scheme



The oxidation seems to be associated with the primary loss of a single hydrogen atom from the nitrogen, rather than with a dehy-

drogenation in the sense of Wieland's theory (Wieland and Bergel (6)), which cannot explain the oxidation of  $\alpha$ -aminoisobutyric acid. The failure of this theory adequately to account for the ready oxidation of  $\alpha$ -dialkylamino acids by oxygen in presence of charcoal has recently been pointed out by Bergel and Bolz (7). The action of silver oxide bears, likewise, only a partial analogy to the oxidation of amino acids (8) in the animal body, for ingested  $\alpha$ -aminoisobutyric acid is excreted unchanged, in substantial entirety, by the dog, without the production of acetone or increased urea in detectable amounts.

Even more striking is the readiness with which  $\alpha$ -dimethylaminoisobutyric acid,<sup>1</sup> in which all the hydrogen atoms of glycine (except that transferred from the carboxyl to the amino group on ionization) are replaced by methyl groups, is oxidized by silver oxide. This compound differed from all others examined in that the nitrogen was liberated (in the form of dimethylamine) more rapidly than the carbon dioxide. To test the possibility that the primary stage of the oxidation consists in hydrolytic deamination, the behavior of  $\alpha$ -hydroxyisobutyric acid under similar conditions was investigated. Carbon dioxide was quantitatively evolved, but at so slow a rate as to preclude the possibility of the hydroxy acid forming an intermediate in the oxidation of either  $\alpha$ -aminoisobutyric acid or its dimethyl derivative.

Acylation of the nitrogen, except in the case of glycine and  $\alpha$ -aminophenylacetic acid, inhibits the oxidizability by silver oxide. Hippuric acid and the benzenesulfonylglycine readily reduce silver oxide without detachment of the acyl groups. Combination of the carboxyl into a peptide linkage prevents oxidation until this linkage is ruptured by hydrolysis. The presence of a dissociated carboxyl group at the  $\alpha$ -carbon atom thus appears to be a necessary factor.

With respect to the hydantoins, our findings parallel those of Baudisch and Davidson (10) in that the substitution of both hydrogen atoms at position (5) (as in 5, 5-dimethylhydantoin) prevents oxidation. Of interest is the formation of a trace of acetylurea from 5-methylhydantoin.

<sup>1</sup> After the completion of the work here reported, Bergel and Bolz (9) have demonstrated the oxidizability of  $\alpha$ -dimethylaminoisobutyric acid by oxygen in presence of charcoal.

## EXPERIMENTAL

*General Procedure*—The first oxidation experiments were carried out by boiling under a reflux a suspension of  $\text{Ag}_2\text{O}$  in an aqueous solution of the amino acid in a slow current of hydrogen;  $\text{NH}_3$  and  $\text{CO}_2$  were absorbed from the gases which escaped from the top of the condenser. In all experiments for which this apparatus was employed, a pronounced lag was encountered in the rate of evolution of  $\text{NH}_3$  as compared with that of  $\text{CO}_2$ . This effect was found to be due to the retention of  $\text{NH}_3$  when a high  $\text{CO}_2$  tension was maintained, and was intensified by passing  $\text{CO}_2$  instead of  $\text{H}_2$  through the apparatus. Since these early experiments were concerned only with the oxidation of glycine, from which 2 moles of  $\text{CO}_2$  are formed for each mole of  $\text{NH}_3$ , the lag in the rate of  $\text{NH}_3$  evolution was not surprising.

In all the experiments described below the apparatus was so modified as to minimize as far as possible the lag in  $\text{NH}_3$  evolution caused by the simultaneous presence of  $\text{CO}_2$ . A 300 cc. Kjeldahl flask was fitted with a 3-hole rubber stopper through which passed an inlet tube for hydrogen, a dropping funnel, and an exit tube leading directly to a declining condenser. The lower end of the condenser was connected to a series of receivers for the absorption of volatile reaction products.

The operation of the apparatus was varied slightly, depending on whether or not the compound to be oxidized was readily soluble in water. In case of amino acids or derivatives easily soluble in cold water, the following general procedure was employed.

Silver oxide, prepared by precipitation from a solution of a weighed amount of silver nitrate with the theoretical amount of 2 N sodium hydroxide, followed by thorough washing with water, was suspended in about 100 cc. of water in the reaction flask and heated to boiling. Throughout the experiment a slow stream of hydrogen was passed through the apparatus. To start the reaction a solution of the amino acid in 25 cc. of water, followed by 25 cc. of wash water, was added to the boiling silver oxide suspension through the dropping funnel, whereupon the absorption train was immediately attached to the condenser.

The oxidation of sparingly soluble compounds was carried out by placing them in the reaction flask, adding silver oxide and cold water, and heating to boiling, care being necessary to avoid ex-

cessive frothing at the outset. The reaction was timed from the first application of heat.

Throughout the experiment the reaction mixture was boiled gently, so as to provide a slow, continuous distillation of water into the first receiver. Water lost by distillation was replaced through the dropping funnel, the volume of liquid being held practically constant throughout the experiment. The absorption train was changed at intervals. The first receiver contained standard acid for the absorption and estimation of volatile base, which, when volatile acids were products of the oxidation, was redistilled from alkaline solution before titration. The second and third receivers contained  $\text{Ba}(\text{OH})_2$  solution for absorption and estimation of  $\text{CO}_2$ . When the absorption train was detached from the apparatus, the first (acid) receiver was always boiled for a few moments in order to drive all the  $\text{CO}_2$  into the  $\text{Ba}(\text{OH})_2$ , before the several receivers were disconnected. The  $\text{BaCO}_3$  was filtered off and washed, dissolved in an excess of standard hydrochloric acid, and titrated.

In several experiments  $\text{Mg}(\text{OH})_2$  was added to the reaction mixture. The liberation of  $\text{CO}_2$  was somewhat restrained, and the rate of  $\text{NH}_3$  evolution was generally more rapid.

When the evolution of  $\text{NH}_3$  and  $\text{CO}_2$  ceased, heating was discontinued, the reaction mixture was filtered, and the residue washed with water. The filtrate and washings were combined for analysis.

The residue was washed with hot dilute acetic acid and hot dilute ammonia solution until free of excess silver oxide and silver salts. The residual metallic silver was then dissolved in nitric acid, and estimated by titration with standard thiocyanate solution.

In the filtrate, the total nitrogen was estimated by the Kjeldahl process, and ammonia nitrogen by distillation with  $\text{Mg}(\text{OH})_2$  or  $\text{NaOH}$ . The acetic acid washings of the silver-silver oxide residues were, as a rule, also subjected to Kjeldahl determinations.

Identifications of other products were made as follows, all melting points being controlled by admixture with authentic samples.

*Methylamine*—Insolubility of hydrochloride in chloroform, and conversion of base into 2,4-dinitromethylaniline, m.p.  $175^\circ$  (uncorrected).

*Dimethylamine*—Solubility of hydrochloride in chloroform, and conversion of base to picrate, m.p.  $158\text{--}159^\circ$  (uncorrected).

**Aniline**—By carbylamine test; occasionally by conversion to acetanilide, m.p. 116–116.5°.

**Azobenzene**—Isolated by extraction of acidified distillate with chloroform, m.p. 67–68°.

**Benzamide**—M.p. 127.5° (uncorrected).

**Benzenesulfonamide**—M.p. 152.5–153° (uncorrected).

**Urea**—Isolated and estimated as compound with xanthidrol, or estimated by urease.<sup>2</sup>

FIG. 1. Glycine (10 mm) and  $\text{Ag}_2\text{O}$  (66 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ ; Curve 3,  $\text{NH}_3$  in presence of  $\text{Mg}(\text{OH})_2$ ; Curve 4,  $\text{NH}_3$  in presence of  $\text{NaOH}$  (50 mm); Curve 5,  $\text{CO}_2$  in presence of  $\text{H}_2\text{SO}_4$  (66 milli-equivalents); Curve 6,  $\text{CO}_2$  in presence of  $\text{CH}_3\text{COOH}$  (66 mm).

FIG. 2. Sarcosine (10 mm) and  $\text{Ag}_2\text{O}$  (66 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{CH}_3\text{NH}_2$ ; Curve 3,  $\text{CH}_3\text{NH}_2$  in presence of  $\text{Mg}(\text{OH})_2$ .

FIG. 3. Dimethylglycine (10 mm) and  $\text{Ag}_2\text{O}$  (66 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $(\text{CH}_3)_2\text{NH}$ ; Curve 3,  $(\text{CH}_3)_2\text{NH}$  in presence of  $\text{Mg}(\text{OH})_2$ ; Curve 4,  $(\text{CH}_3)_2\text{NH}$  in presence of  $\text{NaOH}$  (50 mm).

FIG. 4. Alanine (10 mm) and  $\text{Ag}_2\text{O}$  (50 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ ; Curve 3,  $\text{NH}_3$  in presence of  $\text{Mg}(\text{OH})_2$ . Alanylalanine (5 mm) and  $\text{Ag}_2\text{O}$  (50 milli-equivalents). Curve 4 represents  $\text{CO}_2$ ; Curve 5,  $\text{NH}_3$ .

FIG. 5. N-Methylalanine (10 mm) and  $\text{Ag}_2\text{O}$  (50 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{CH}_3\text{NH}_2$ ; Curve 3,  $\text{CH}_3\text{NH}_2$  in presence of  $\text{Mg}(\text{OH})_2$ .

FIG. 6. N-Dimethylalanine (10 mm) and  $\text{Ag}_2\text{O}$  (50 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $(\text{CH}_3)_2\text{NH}$ ; Curve 3,  $(\text{CH}_3)_2\text{NH}$  in presence of  $\text{Mg}(\text{OH})_2$ .

FIG. 7. Leucine (10 mm) and  $\text{Ag}_2\text{O}$  (70 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ ; Curve 3,  $\text{NH}_3$  in presence of  $\text{Mg}(\text{OH})_2$ .

FIG. 8.  $\alpha$ -Aminoisobutyric acid (10 mm) and  $\text{Ag}_2\text{O}$  (30 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ ; Curve 3,  $\text{NH}_3$  in presence of  $\text{Mg}(\text{OH})_2$ .

FIG. 9.  $\alpha$ -Dimethylaminoisobutyric acid (10 mm) and  $\text{Ag}_2\text{O}$  (30 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $(\text{CH}_3)_2\text{NH}$ .  $\alpha$ -Hydroxyisobutyric acid (10 mm) and  $\text{Ag}_2\text{O}$  (60 milli-equivalents). Curve 3 represents  $\text{CO}_2$ .

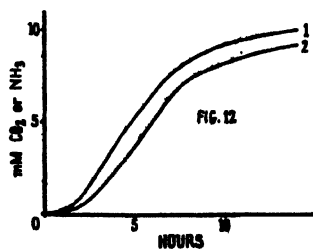
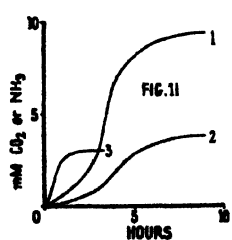
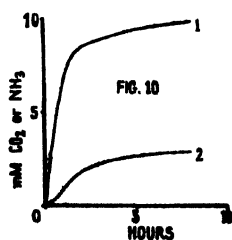
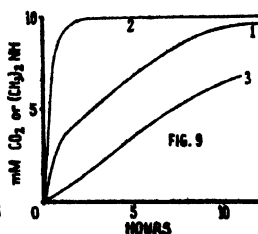
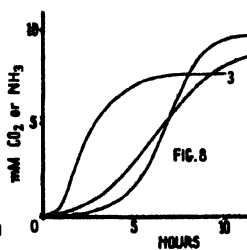
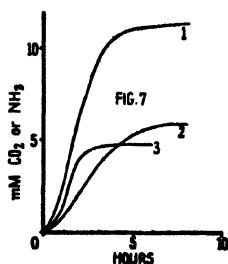
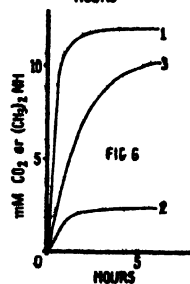
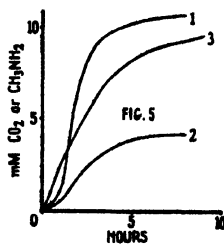
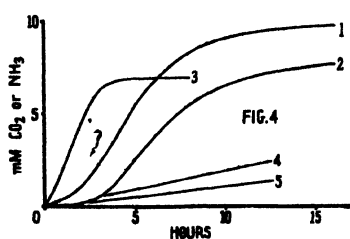
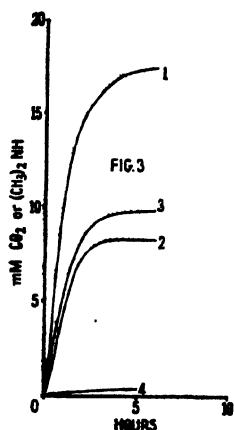
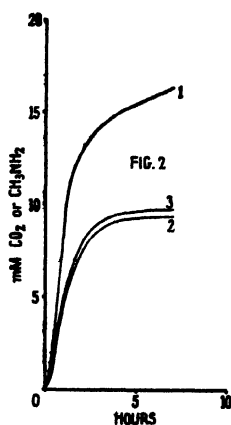
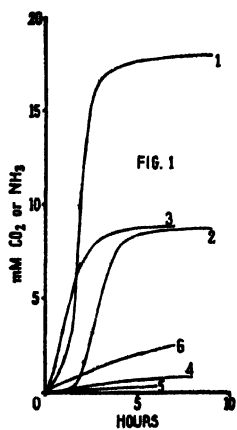
FIG. 10.  $\alpha$ -Aminophenylacetic acid (10 mm) and  $\text{Ag}_2\text{O}$  (60 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ .

FIG. 11. Phenylalanine (5 mm) and  $\text{Ag}_2\text{O}$  (60 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ . Phenylacetic acid (5 mm) and  $\text{Ag}_2\text{O}$  (36 milli-equivalents). Curve 3 represents  $\text{CO}_2$ .

FIG. 12.  $\alpha$ -Amino- $\alpha$ -phenyl-*n*-butyric acid (10 mm) and  $\text{Ag}_2\text{O}$  (40 milli-equivalents). Curve 1 represents  $\text{CO}_2$ ; Curve 2,  $\text{NH}_3$ .

<sup>2</sup> The authors are indebted to Dr. Samuel Graff and Miss Rhoda Howard for these determinations.





FIGS. 1 to 12

*Acetaldehyde*—As derivative of dimethyldihydroresorcinol, m.p. 143–144° (uncorrected).

*Acetone*—As 2,4-dinitrophenylhydrazone, m.p. 126–128° (uncorrected).

*Isovaleraldehyde*—As derivative of dimethyldihydroresorcinol, m.p. 154–155° (uncorrected).

*Benzaldehyde*—As *p*-nitrophenylhydrazone, m.p. 204–205° (uncorrected).

*Acetic Acid*—Separated by distillation from dilute phosphoric acid, isolated as silver acetate (Ag found, 64.2 to 64.5; calculated, 64.6).

*Benzoic Acid*—M.p. 123° (uncorrected).

In all of the quantitative experiments, the results of which are outlined below, complete accounts of the nitrogen and silver distribution were kept; records of these are withheld for economy of space.

*Glycine (Fig. 1)*—In Fig. 1 are shown typical curves for the rate of evolution of  $\text{NH}_3$  and  $\text{CO}_2$  during the oxidation of glycine. Curve 3 was obtained in the presence of  $\text{Mg}(\text{OH})_2$ , and shows a slight increase in the rate of  $\text{NH}_3$  evolution. Curves 4, 5, and 6 show the effect of sodium hydroxide, sulfuric acid, and acetic acid respectively on the rate of oxidation of glycine by silver oxide.

On oxidation with silver oxide under the conditions described above, the main products obtained from glycine are  $\text{CO}_2$  and  $\text{NH}_3$ . The yield of silver varied from 5.7 to 6.4 atoms per mole of glycine. There was generally a loss of 10 to 15 per cent of the nitrogen, presumably in the form of  $\text{N}_2$ .

The only other product obtained as a result of oxidation was a small amount of urea. A suspension of silver oxide in an aqueous solution of glycine was boiled for 2 hours under a reflux. A current of hydrogen through the apparatus washed the gaseous reaction products into suitable receivers attached to the upper end of the condenser. The reaction mixture was cooled to room temperature as rapidly as possible at the end of 2 hours.<sup>3</sup> In two such experiments, the yields of  $\text{CO}_2$  were 1.1 and 1.2 moles, of  $\text{NH}_3$  0.82

<sup>3</sup> At this point, in several other experiments, aniline hydrochloride was added. In no case was evidence of the formation of phenylurea obtained, although urea itself was always found. Cyanate cannot, therefore, have been present in significant amounts.

and 0.82 moles, and of metallic silver 6.0 and 6.2 atoms, per mole of glycine. The cold reaction mixture was filtered, and an aliquot of the filtrate was treated with  $H_2S$  to remove silver, and then concentrated to a small volume for the estimation of urea, in which form 5 to 8 per cent of the nitrogen was recovered.

*Sarcosine* (Fig. 2)—The same general formulation as written for glycine can be applied to the oxidation of sarcosine, except that

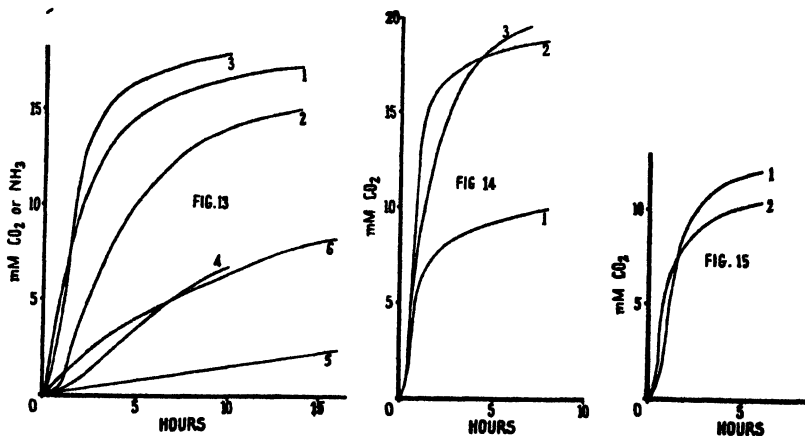


FIG. 13.  $\alpha$ -Ureidopropionic acid (10 mM) and  $Ag_2O$  (80 milli-equivalents). Curve 1 represents  $CO_2$ ; Curve 2,  $NH_3$ . 5-Methylhydantoin (10 mM) and  $Ag_2O$  (80 milli-equivalents). Curve 3 represents  $CO_2$ ; Curve 4,  $NH_3$ . Urea (10 mM) and  $Ag_2O$  (10 milli-equivalents). Curve 5 represents  $CO_2$ ; Curve 6,  $NH_3$ .

FIG. 14. Hippuric acid (10 mM) and  $Ag_2O$  (76 milli-equivalents). Curve 1 represents  $CO_2$ . Benzenesulfonylglycine (10 mM) and  $Ag_2O$  (80 milli-equivalents). Curve 2 represents  $CO_2$ . N-Phenylglycine (10 mM) and  $Ag_2O$  (100 milli-equivalents). Curve 3 represents  $CO_2$ .

FIG. 15. N-Phenylalanine (10 mM) and  $Ag_2O$  (80 milli-equivalents). Curve 1 represents  $CO_2$ .  $\alpha$ -Phenylaminoisobutyric acid (10 mM) and  $Ag_2O$  (40 milli-equivalents). Curve 2 represents  $CO_2$ .

the nitrogen was recovered almost quantitatively in the form of methylamine; 5.9 to 6.2 atomic proportions of metallic silver were produced.

*Dimethylglycine* (Fig. 3)—This was employed as the hydrochloride, a correspondingly increased amount of silver oxide being taken. No attempt was made to remove silver chloride prior to the reaction, owing to the readiness with which dimethylglycine is oxidized

by silver oxide, even in the cold. The yield of silver was 6.5 to 6.6 atomic proportions. No appreciable loss of nitrogen occurred, all of it reappearing as dimethylamine. As with glycine, the reaction is almost completely inhibited by sodium hydroxide.

*N-Phenylglycine* (Fig. 14)—Owing to the character of the volatile oxidation products (aniline and azobenzene) obtained from this amino acid, the condenser was replaced by a flask cooled with running water. The delivery tube passed directly into the cooled receiver, containing dilute acid for the absorption of basic reaction products, which were collected continuously throughout the reaction. The receiver for carbon dioxide was changed at intervals.

After extracting the azobenzene (0.13 equivalent of nitrogen) from the distillate, the aniline (0.28 equivalent) was estimated by the Kjeldahl process. The filtrate from the reaction mixture and the acetic acid washings of the silver-silver oxide residue were both colored reddish purple, and together contained 0.59 equivalent of nitrogen. The value for silver (6.9 atoms) was high, owing to difficulty in washing the residue.

A mixture of silver oxide and aniline in water was distilled in the above apparatus. Azobenzene appeared in the first few drops of distillate, together with some aniline; intensely colored oxidation products were also formed.

*Hippuric Acid* (Fig. 14)—No nitrogen was liberated, either as  $\text{NH}_3$  or  $\text{N}_2$ ; all was recovered as benzamide in the filtrate from the reaction mixture. Only about one-half the theoretical amount (1.0 to 1.1 moles) of  $\text{CO}_2$  was liberated, and 4.5 to 4.7 atomic proportions of silver were formed. These low values require explanation; possible intermediates such as glyoxylic acid and oxalic acid were sought in vain.

*Benzenesulfonylglycine* (Fig. 14)—In analogy with the case of hippuric acid, benzenesulfonamide was formed during the oxidation. No nitrogen was liberated as  $\text{N}_2$  or  $\text{NH}_3$ , all being recovered in the filtrate and the acetic acid washings of the residue. The amounts of carbon dioxide (1.86 to 1.88 moles) and of silver formed (6.0 to 6.1 atoms) agreed closely with the theoretical values.

*Alanine* (Fig. 4)—Besides ammonia and carbon dioxide, acetaldehyde and acetic acid (0.6 to 0.8 mole) were isolated as reaction products. Varying amounts of nitrogen (0.16 to 0.26 equivalent) disappeared during the oxidation, presumably as  $\text{N}_2$ ; only traces

of urea were found. The yield of silver was 4.0 atoms in two experiments carried out in water alone; in the presence of magnesia, 4.7 atoms were obtained.

*N-Methylalanine (Fig. 5)*—The reaction was more rapid than with alanine. Some of the base was held back during the reaction, probably by the resulting acetic acid, since it was easily released on distillation with magnesia. No loss of nitrogen occurred; all reappeared as methylamine. Acetaldehyde was present only in small quantity, the major portion having been oxidized to acetic acid (0.88 mole); 4.6 to 4.9 atoms of silver were formed.

*N-Dimethylalanine (Fig. 6)*—The oxidation was slightly more rapid than that with methylalanine, and followed the same course, yielding carbon dioxide (1.2 moles), dimethylamine (1.1 to 1.2 moles), acetic acid (0.85 to 1.0 mole), a trace of acetaldehyde, and silver (5.0 atoms in two experiments).

*N-Phenylalanine (Fig. 15)*—The thermal instability of the silver salt of this compound has been noted by Tiemann and Stephen (11).

The oxidation was carried out as described for N-phenylglycine, and gave analogous results, aniline (0.21 mole), azobenzene (0.17 equivalent of N), CO<sub>2</sub> (1.2 moles), and acetic acid (0.69 mole) being the chief products. Owing to difficulty in washing, the yield of silver (6.7 atoms) was unduly high. A deficiency of about 6 per cent of the nitrogen may be due to the same cause.

*α-Ureidopropionic Acid (Fig. 13)*—This compound, in contrast to the acylated alanine derivatives, was readily oxidized by a boiling suspension of silver oxide, with formation of 1.7 to 1.9 moles of carbon dioxide and 1.35 to 1.68 moles of ammonia. Urea was formed only in traces. Judging by the rate of evolution of CO<sub>2</sub> and NH<sub>3</sub>, urea does not enter as an important intermediate step, since both were liberated in this reaction much more rapidly than they are from urea under the same conditions (Fig. 13, Curves 5 and 6). Acetaldehyde was formed in considerable amounts, together with acetic acid (0.5 to 0.6 mole). The yield of silver was 4.5 to 4.7 atoms.

*5-Methylhydantoin (Fig. 13)*—As in the case of α-ureidopropionic acid, a nearly quantitative yield (1.8 moles) of carbon dioxide was obtained. Ammonia (0.68 mole) was formed, but much of the nitrogen (1.1 equivalents) was retained in the reaction mixture.

Urea was present only in traces (0.05 equivalent of N). No trace of acetaldehyde could be found; 0.92 mole of acetic acid was recovered. The yield of silver was 5.7 atoms. From the filtrate there was isolated a minute amount of a crystalline solid, m.p. 213–214° (uncorrected). This appeared to consist of acetylurea.

*Analysis*

Calculated for  $C_2H_4O_2N_2$ . C 35.29, H 5.88, N 27.4

Found. " 35.66, " 5.87, " 27.6 (Kjeldahl), 24.8 (Dumas)

*Alanylalanine* (Fig. 4)—Alanylalanine was oxidized only very slowly by silver oxide; after 14 hours of boiling, 0.25 mole of ammonia, 0.48 mole of carbon dioxide, 0.18 mole of acetic acid, and 2.1 atoms of silver were formed. There was no loss of nitrogen; unchanged alanylalanine was recovered.

*Leucine* (Fig. 7)—Rapid oxidation occurred, with production of  $CO_2$ ,  $NH_3$ , and isovaleraldehyde. The large loss of nitrogen (0.23 to 0.40 equivalent), particularly in presence of magnesia (0.45 equivalent), is noteworthy. Isovaleric acid was not isolated, but its presence was evident from the odor. Further oxidation of isovaleric acid by silver oxide does not take place to any great extent, as was proved by direct experiment, and could be inferred from the proximity of the yield of  $CO_2$  (1.1 moles in two experiments) to the theoretical value. The amount of silver formed in each case (4.8 to 5.4 atoms) was only slightly more than that required to bring about the primary reaction and to oxidize the missing  $NH_3$  to  $N_2$ .

*$\alpha$ -Aminoisobutyric Acid* (Fig. 8)—Aminoisobutyric acid was oxidized at about the same rate as alanine, with formation of  $CO_2$ ,  $NH_3$ , and acetone. Of interest are the loss of nitrogen (0.16 equivalent) which occurred only in the presence of magnesia, and the fact that, although  $NH_3$  was evolved more slowly than  $CO_2$  during the early stages of the reaction, it is subsequently released more rapidly. The yields of silver were 2.1 atoms in the absence of magnesia, 2.9 in its presence.

An experiment in which  $\alpha$ -hydroxyisobutyric acid was oxidized with silver oxide (Fig. 9) showed definitely, by the much slower rate of  $CO_2$  evolution, that this compound cannot have been formed as an intermediate stage in the oxidation of the corresponding amino acid.

*$\alpha$ -Dimethylaminoisobutyric Acid (Fig. 9)*—The oxidation of this compound (1) was slower in the cold than that of dimethylglycine, but was slightly more rapid in hot solution. The relative rates of evolution of base and  $\text{CO}_2$  during the oxidation of dimethylaminoisobutyric acid differ from the others in that dimethylamine is evolved far more rapidly than the  $\text{CO}_2$ . The formation of acetone was established only qualitatively; according to Linnemann (12) acetone is appreciably oxidizable by silver oxide, a fact which may explain the rather high yield (2.1 to 2.3 atoms) of silver. There was no loss of nitrogen.

*$\alpha$ -N-Phenylaminoisobutyric Acid (Fig. 15)*—Although the heat instability of the silver salt (13) of N-phenylaminoisobutyric acid has long been known, nothing concerning the oxidation of this acid appears in the literature.

The reaction was carried out in the apparatus employed for the oxidation of N-phenylglycine. Aniline and azobenzene, formed in yields of 0.64 and 0.04 equivalents respectively, were both present in the first drops of distillate, which also contained acetone. The filtrate from the reaction mixture and the acetic acid washings of the silver-silver oxide residue were colored intensely reddish purple, and contained 0.27 equivalent of nitrogen. It was impossible to wash the residue free of all organic material; this may account for a loss of about 5 per cent of the nitrogen. The yield of silver was 3.1 atoms.

*$\alpha$ -Aminophenylacetic Acid (Fig. 10)*—Rapid oxidation occurred, with formation of  $\text{CO}_2$ ,  $\text{NH}_3$ , benzaldehyde, and benzoic acid. The yield of silver was 4.5 to 5.0 atoms. Most striking is the loss of 40 to 50 per cent of the nitrogen during the reaction. An experiment devised to demonstrate the formation of  $\text{N}_2$  during the oxidation of aminophenylacetic acid is described below.

*Phenylalanine (Fig. 11)*—Oxidation was slightly more rapid than with alanine; approximately 2 moles of  $\text{CO}_2$  were formed for every mole of  $\text{NH}_3$ , indicating almost complete oxidation of the side chain. The distillate contained benzaldehyde, contaminated by a trace of phenylacetaldehyde (discernible by its odor); benzoic acid, containing a trace of phenylacetic acid (detected by its odor), was formed in larger proportion. The yield of silver was 10.6 atoms; a loss of about 10 per cent of the nitrogen occurred.

An experiment with phenylacetic acid (Fig. 11) indicated fairly

ready oxidizability by silver oxide, with formation of benzoic acid and  $\text{CO}_2$ . As the reaction was carried out under a reflux, no benzaldehyde was isolated.

*$\alpha$ -Amino- $\alpha$ -Phenyl-*n*-Butyric Acid* (Fig. 12)—Oxidation took place with formation of  $\text{CO}_2$ ,  $\text{NH}_3$ , and propiophenone, identified as its semicarbazone, m.p.  $177$ – $178^\circ$  (uncorrected). No loss of nitrogen occurred; the yield of silver was 2.7 atoms.

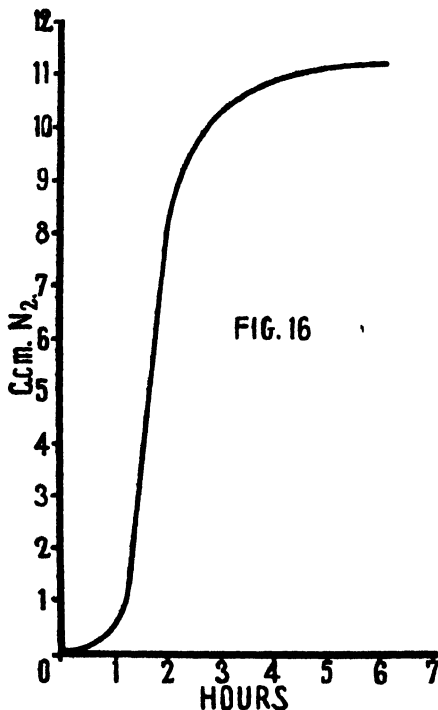


FIG. 16.  $\text{N}_2$  formed from  $\alpha$ -aminophenylacetic acid (10 mm) and  $\text{Ag}_2\text{O}$  (60 milli-equivalents).

*Formation of Nitrogen during Oxidation of  $\alpha$ -Aminophenylacetic Acid*—In various experiments on the oxidation of  $\alpha$ -aminophenylacetic acid with  $\text{Ag}_2\text{O}$ , as much as 40 to 50 per cent of the nitrogen disappeared. This amino acid, therefore, seemed particularly suitable for an experiment designed to test the formation of molecular nitrogen during the course of oxidation.



In this experiment the reaction was carried out under a reflux, in a slow current of carbon dioxide. The gaseous reaction products were washed with dilute sulfuric acid, collected over 40 per cent potassium hydroxide, and measured periodically, corrections being made for the values observed in blank experiments. Fig. 16 shows the amounts of nitrogen liberated from a solution of 1.51 gm. (10 mm) of  $\alpha$ -aminophenylacetic acid in 150 cc. of water on boiling with the freshly precipitated silver oxide from 10.2 gm. (80 mm) of silver nitrate. At the end of the reaction, 7.52 milli-equivalents of nitrogen were present in the residual solution, 0.04 in the acid wash liquor. The total volume of nitrogen gas evolved was 11.2 cc. ( $0^\circ$  at 760 mm.) or 1.00 milli-equivalent, bringing the total recovery up to 85.6 per cent of the theoretical amount.

*Metabolism of  $\alpha$ -Aminoisobutyric Acid*—A dog weighing 22 kilos was placed on a suitable diet supplying a constant daily nitrogen intake of 13.3 gm., water being allowed *ad libitum*. The urine, collected in 3 day periods, was analyzed for total nitrogen, urea, and ammonia. After 15 days, three daily portions of 20 gm. of  $\alpha$ -aminoisobutyric acid were added to the diet. After this period, the standard ration was resumed for 6 days.

No significant change in weight occurred throughout the experiment. During the first 2 days on which the amino acid was fed, the dog was extremely thirsty and drank abnormally large quantities of water, with a corresponding increase in urine volume. This effect was not noted on the 3rd day.

From Fig. 17 it will be seen that the urea nitrogen did not rise during the period of amino acid feeding, nor were there significant changes in ammonia nitrogen. Attempts to estimate amino nitrogen were frustrated by the observation that  $\alpha$ -aminoisobutyric acid differs from the naturally occurring amino acids (13) in its response to the Folin procedure (14); the color develops more slowly and reaches a final intensity of only about one-third of the usual value.

It is clear that the aminoisobutyric acid was not metabolized by the dog. This conclusion is supported by the isolation of the unchanged amino acid in a yield of 75 per cent from the urine collected during the experimental period: To 1 liter of urine, representing 16.7 gm. of ingested aminoisobutyric acid, lead acetate (30 gm.) was added until no further precipitation

occurred. The filtrate and washings were treated with 13 gm. of sodium bicarbonate, the precipitate removed, 50 cc. of 25 per cent sodium hydroxide were added, and the solution evaporated in a copper vessel over a free flame. When the volume had been reduced to 100 cc., 60 gm. of crystallized barium hydroxide were added, and the mixture heated in an oven at 115° for 21 hours. The residue was taken up in water, the barium carbonate was filtered off, and the filtrate boiled in the presence of 1 gm. of zinc filings until its temperature reached 105°. It was then replaced

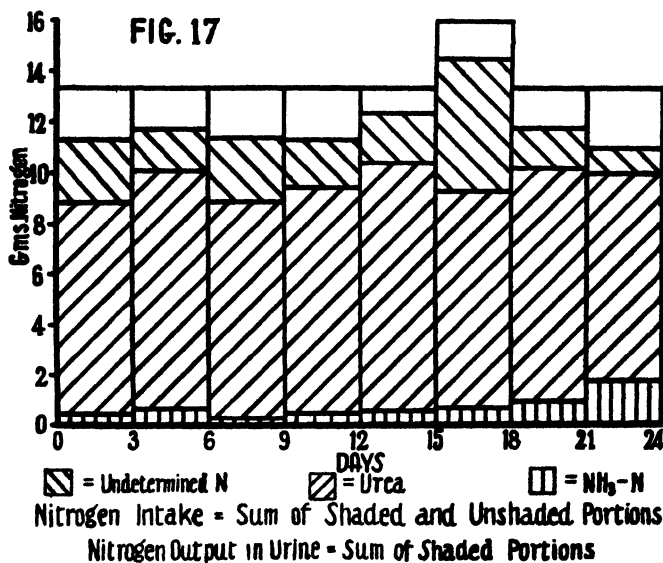


FIG. 17. Nitrogen distribution in urine on feeding  $\alpha$ -aminoisobutyric acid.

in the oven for 20 hours. The residue was dissolved, filtered, and freed of heavy metals by hydrogen sulfide. The filtrate was acidified to litmus with hydrochloric acid, filtered, shaken with ether to remove fatty acids, cleared with norit, acidified to Congo red with hydrochloric acid, and evaporated to dryness. The residue was extracted with absolute alcohol, and the soluble fraction recrystallized from glacial acetic acid. Mother liquors were worked up systematically. There were thus obtained 16.9 gm. of pure, crystalline  $\alpha$ -aminoisobutyric acid hydrochloride (m.p. and mixed m.p.

253°), corresponding to 12.5 gm. (75 per cent of the theoretical amount) of the free amino acid. The phenylureide had a melting point and a mixed melting point of 174°. No attempt was made to estimate the amino acid present in the non-crystalline residue, which amounted to less than 1 gm.

In similar experiments with 1 liter quantities of urine from the preexperimental period, with and without added  $\alpha$ -aminoisobutyric acid, the alcohol-soluble residues were analyzed without attempting to secure crystalline fractions. The results, recorded in Table II, indicate the recovery of about 77 per cent of the calculated amount of amino nitrogen under the conditions adopted. In the determinations of amino acid nitrogen by Folin's method,  $\alpha$ -aminoisobutyric acid was employed as standard.

TABLE II

*Nitrogen Distribution in Alcohol-Soluble Fractions of Urine after Successive Treatment with Alkali and Hydrochloric Acid*

	Total N	Urea N	Amino N Van Slyke	Amino acid N Folin
	mg.	mg.	mg.	mg.
1 liter urine (preexperimental) .....	155	1.3	93	65
Same + 1.0 gm. $\alpha$ -aminoisobutyric acid .. . . . . .	347	7.7	198	172

## SUMMARY

$\alpha$ -Amino acids are oxidized in hot aqueous solution by silver oxide, with formation of ammonia, carbon dioxide, and aldehydes; the latter may undergo further oxidation to the corresponding acids. Replacement of both amino hydrogen atoms by methyl groups facilitates rather than inhibits the oxidizability, but in betaine this faculty is entirely lost. The presence of hydrogen at the  $\alpha$ -carbon atom is not essential to oxidizability. Acylation of the amino group tends to prevent oxidation, but this effect is by no means universal.

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